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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

REFINING THE EVALUATION OF GROWTH AND THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS IN CHILDREN

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TABLE OF CONTENTS

DECLARATION OF OWNERSHIP

This thesis submitted is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Colin Patrick Hawkes

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LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxvitamin D
25(OH)D	25 hydroxyvitamin D
ADP	Air Displacement Plethysmography
ADHD	Attention deficit and hyperactivity disorder
AGA	Appropriate for Gestational Age
AIDS	Acquired Immune Deficiency Syndrome
ALS	Acid Labile Subunit
AKT	Protein Kinase B
BASELINE	Babies After Scope; Establishing the Longitudinal Impact using Neurological and nutritional Endpoints
BCH	Boston Children's Hospital
%BF	Percentage Body Fat
BIA	Bioelectric Impedance Analysis
BMI	Body Mass Index
CCHMC	Cincinnati Children's Hospital Medical Center
CHOP	Children's Hospital of Philadelphia
CYP	Cytochrome P450
D_2	Ergocalcifierol
D_3	Cholecalciferol
DXA	Dual-energy x-ray absorptiometry
ECL	Enzyme-linked Chemiluminescent Assay
EHR	Electronic Health Record
ELISA	Enzyme-linked Immunosorbent Assay
ESR:	Erythrocyte Sedimentation Rate
FFM	Fat Free Mass
FFMI	Fat Free Mass Index
FGF21	Fibroblast Growth Factor 21
FM	Fat Mass
FMI	Fat Mass Index
GH	Growth Hormone

GHBP	Growth Hormone Binding Protein
GHD	Growth Hormone Deficiency
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GHST	Growth Hormone Stimulation Test
GRIN	Genomics Research and Innovation Network
GRP94	Glucose Regulated Protein 94
GTP	Guanosine Triphosphate
HV	Height velocity
IGF-I	Insulin-like Growth Factor-I
IGFBP	Insulin-like Binding Protein
IGFBP-3	Insulin-like Binding Protein-3
ITT	Insulin Tolerance Test
JAK	Janus Kinase
L	Length
LCMS	Liquid Chromatography / Mass Spectometry
LMP	Last Menstrual Period
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK Kinase
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic Target of Rapamycin
NCGS	National Cooperative Growth Study
NPY	Neuropeptide Y
NSD	Neurosecretory Dysfunction
OLCHC	Our Lady's Children's Hospital Crumlin
PI3K	Phosphoinositide-3-kinase
PTH	Parathyroid hormone
RIA	Radioimmunoassay
Ral	Ras-related protein
SCOPE	Screening for Pregnancy Endpoints
SGA	Small for Gestational Age
SOCS2	Suppressor of Cytokine Signaling 2

SH2	Src Homology
STAT	Signal Transducer and activator of transcription
T1D	Type 1 Diabetes
VDR	Vitamin D Receptor
WHO	World Health Organization

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

PEER-REVIEWED PUBLICATIONS

1. **Hawkes CP**, Mavinkurve M, Fallon M, Grimberg A, Cody DC. Serial GH measurement after intravenous catheter placement alone can detect levels above stimulation test thresholds in children. J Clin Endocrinol Metab. 2015;100(11):4357-63. (Appendix B)

2. **Hawkes CP**, Grimberg A, Dzata VE, De Leon DD. Adding glucagon-stimulated GH testing to the diagnostic fast increases the detection of GH-sufficient children. Horm Res Paediatr. 2016;85(4):265-72. (Appendix D)

3. **Hawkes CP**, Grimberg A. Measuring growth hormone and insulin-like growth factor-I in infants: what is normal? Pediatr Endocrinol Rev. 2013;11(2):126-46. (Appendix F)

4. **Hawkes CP**, Murray DM, Kenny LC, Kiely M, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Correlation of insulin-like growth factor-I and –II concentrations at birth measured by mass spectrometry and growth from birth to two months. Horm Res Paediatr. 2018 Jan. doi 10.1159/000486035 [Epub ahead of print]. (Appendix I)

5. Marzec M, **Hawkes CP**, Eletto D, Boyle S, Rosenfeld R, Hwa V, Wit JM, van Duyvenvoorde HA, Oostdijk W, Losekoot M, Pedersen O, Beng Yeap B, Flicker L, Barzilai N, Atzmon G, Grimberg A, Argon Y. A human variant of glucose-regulated protein 94 that inefficiently supports IGF production. Endocrinology. 2016;157(5):1914-28. (Appendix J)

6. **Hawkes CP**, Grimberg A. Insulin-like growth factor-I is a marker for the nutritional state. Pediatr Endocrinol Rev. 2015;13(2):465-77. (Appendix K)

7. **Hawkes CP**, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Gender- and gestational age-specific body fat percentage at birth. Pediatrics. 2011;128(3):E645-E51. (Appendix L)

8. **Hawkes CP**, Zemel BS, Kiely M, Irvine AD, Kenny LC, O'B Hourihane J, Murray DM. Body composition within the first 3 months: optimized correction for length and correlation with BMI at 2 years. Horm Res Paediatr. 2016;86(3):178-187. (Appendix N)

MANUSCRIPTS UNDER REVIEW

1. **Hawkes CP**, Zemel BS, Kenny LC, Kiely M, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life.

BOOK CHAPTERS

1. **Hawkes CP**, Stanley CA. Pathophysiology of neonatal hypoglycemia. In: Polin RA, Fox WW, Abman SH, editors. Fetal and Neonatal Physiology: Expert Consult - Online and Print. 5 ed: Elsevier/Saunders; 2016. p. 1550-60. (Appendix C)

PRESENTATIONS AT INTERNATIONAL MEETINGS

1. **Hawkes CP**, Grimberg A, Dzata VE, De Leon DD. Integrating growth hormone testing with hypoglycaemia investigation. American Pediatric Society / Society for Pediatric Research, May 2014. (Poster Presentation). (Appendix E)

2. **Hawkes CP**, Murray DM, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Measurement of IGF-I and –II concentrations at birth by mass spectrometry in a large birth cohort: correlation with anthropometry. Pediatric Endocrine Society, Washington DC. September 2017 (Poster Presentation) (Appendix H)

3. **Hawkes CP**, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Body Composition at birth; normative values. American Pediatric Society / Society for Pediatric Research. Denver, May 2011 (Poster Presentation). European Society for Paediatric Research, Newcastle, Sept 2011 (Poster Presentation). (Appendix M)

4. **Hawkes CP**, Zemel BS, Kiely M, Irvine A, Kenny LC, O'B Hourihane J, Murray DM. Body composition in the first 2 months of life – optimized correction for length, reference data and correlation with obesity at 2 years. American Pediatric Society / Society for Pediatric Research. Baltimore, May 2016 (Poster Presentation). (Appendix O)

5. **Hawkes CP**, Zemel BS, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life. Pediatric Endocrine Society, September 2017 (Poster Presentation). (Appendix P)

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ABSTRACT

Introduction

The growth hormone (GH)/Insulin-like growth factor-I (IGF) axis is a key mediator of childhood growth. Current diagnostic tests have poor specificity for disorders affecting this system, namely the growth hormone stimulation test (GHST) and IGF-I measurement. Furthermore, advances in genetics and body composition analysis may provide new approaches to diagnosing disorders involving this axis.

Aim

To improve the diagnostic evaluation of children with poor growth and possible GH deficiency (GHD) through novel approaches to 1) modifying the GHST and diagnostic fasting study; 2) utilising liquid chromatography mass spectrometry (LCMS) to measure IGF-I and –II concentrations; 3) exploring rare genetic causes of poor growth; and 4) studying the association between early body composition and early infant growth.

Methods

I used various approaches including: additional timed GH measurements during the diagnostic GHST and fasting study; IGF-I and –II measurement by LCMS in a well-characterised birth cohort; population-based screening for genetic polymorphisms; focused whole exome testing for rare clinical phenotypes; and body composition analysis measured using air displacement plethysmography.

Results

Intravenous line placement (IVP) is a stimulus for GH secretion and serial additional GH measurement after placement will improve the specificity of the GHST. Similarly, serial measurement of GH in the context of a diagnostic fasting study will improve the specificity for disease. Nutrition interacts with the GH/IGF axis, and I have described reference data for body composition to improve measurement of nutritional status in infancy. In normal infants, there is

a doubling of body fat in the first two months. Using liquid chromatography mass spectrometry, I have described reference data for IGF-I and –II at birth and demonstrated a relationship between these measurements and this rapid accumulation body fat in early infancy. Using a rare phenotype approach and chart review to identify potential genetic causes of short stature, we have also identified a novel *IGF1R* mutation in a child with a phenotype consistent with IGF-I resistance.

Conclusions

Diagnosing disorders of the GH/IGF-I axis remain a significant clinical challenge. I have expanded the clinical approach to evaluating the child with short stature through refining the approaches to the GHST, diagnostic fasting study and body composition evaluation. I have also described reference data for body composition and IGF-I and –II in infancy, and explored potential novel genetic causes of disordered growth. Future work will focus on studying other clinical tools in evaluating the child with short stature and predicting the clinical response to GH treatment.

OVERVIEW OF THIS THESIS

AIM OF THESIS

The aim of this thesis is to refine the diagnostic approaches to normal and abnormal growth in childhood, and disorders of the GH / IGF-I axis.

STRUCTURE

Section 1: The Diagnosis of Growth Hormone Deficiency

In the first section, I will explore the potential of modifications to the GHST to improve specificity for GHD. This will focus separately on children with possible GHD undergoing insulin tolerance tests (ITT), and on children with unexplained hypoglycemia undergoing diagnostic fasting studies.

Section 2: Mass Spectrometry and Insulin-like Growth Factor-I Measurement

GH mediates systemic and local IGF-I production, and IGF-I concentrations are more consistent in serum than GH. Thus, IGF-I levels are often used as a screening tool for GHD[1, 2]. However, IGF-I measurement has limitations, most notably interference in measurement from binding proteins. Radioimmunoassays have traditionally been used to measure IGF-I concentrations but LCMS represents an opportunity to remove this interference from IGF-I measurement. In the second section of this thesis, I will review the literature and describe a study in which we used LCMS to measure serum IGF-I levels in a pediatric birth cohort.

Section 3: Genetic Approaches to Disorders of the GH / IGF-I Axis

There have been significant advances in the molecular and genetic understanding of the GH/IGF-I axis in recent years. In the third section of this thesis, I will focus on genetic approaches to improving our understanding of disorders of this axis. Given of the rarity of these disease-causing mutations, multi-centre collaboration is required to identify and study subjects with specific mutations. I

will describe a study in which separate collaborations were formed to demonstrate the pathogenicity of one common population variant, and a separate study to identify mutations causing a rare clinical phenotype. Although many investigators were involved in each of these studies, I will highlight my specific contributions to these bodies of work.

Section 4: Nutrition, Growth and the GH / IGF-I Axis

In the fourth section of this thesis, I will focus on the role of nutrition in childhood growth and the interaction between this and the GH / IGF-I axis. In addition to reviewing the interaction between nutrition and the GH/IGF-I axis, I will investigate the changes in body composition in early infancy and how this related to growth. The interplay between these new reference data for body composition in infancy with IGF-I and IGF-II measurement will also be described in this section.

Conclusion of Thesis

I will conclude this thesis with a summary of the contributions that this research has made to the evaluation of children with possible disorders of the GH / IGF-I axis. I will also provide an overview of my own personal development over the course of this thesis, and describe a number of active research studies that are building on this work.

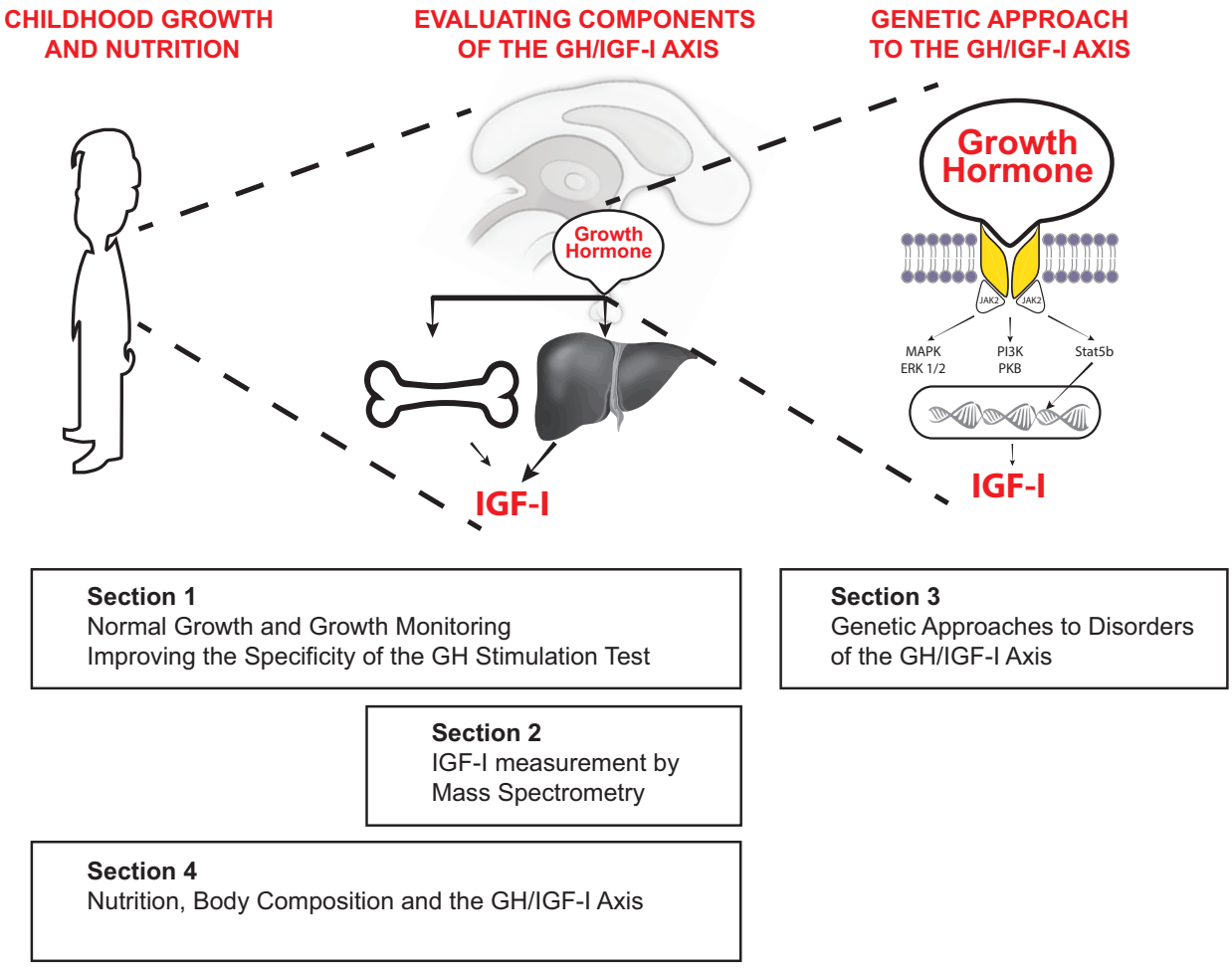

Figure 1.1: This thesis evaluates three different approaches to evaluating childhood growth. This includes: Systemic (measurement and nutrition); Hormonal evaluation of the GH/IGF-I axis; and Genetic approaches.
GH=Growth Hormone, IGF-I=Insulin-like Growth Factor-I.

SECTION 1

DIAGNOSING GROWTH HORMONE DEFICIENCY IN CHILDHOOD

CHAPTER 1.1: INTRODUCTION

Growth in childhood is a dynamic process that is dependent upon genetic, psychosocial, nutritional, and medical factors. Disruption of any of these factors can affect growth, and deviations from normal growth patterns may consequently be the initial presenting sign of a long list of potential diagnoses. Thus, the monitoring of childhood growth is a crucial component of routine paediatric care and represents a valuable screening tool for detecting disease[3-5].

The growth hormone (GH) / insulin-like growth factor (IGF)-I axis is a key mediator of childhood growth, and disordered GH secretion or action is considered as a possible cause of short stature when systemic disease has been ruled out[6]. Despite human GH replacement therapy being available since 1956 and synthetic GH being produced since 1978[7], the diagnostic approach to GH deficiency (GHD) is limited by tests that have poor specificity for disease[8, 9]. For this reason, GHD has been described as "the most difficult condition to diagnose, but easiest to treat"[10]. Deviations from the expected growth pattern may be an early sign of disease, but can also represent measurement error or normal variants of childhood growth[6, 11]. This will be reviewed in Chapter 1.2.

Variation in the diagnostic approaches to GHD is a direct result of the absence of a suitable "gold standard" test. The growth hormone stimulation test (GHST) is widely used, but poorly specific for disease. This is also true for the random measurement of GH during hypoglycaemia[12], despite erroneously being described as a "quick and definitive diagnostic tool" in this setting[13]. In Chapters 1.3 and 1.4, I will describe studies in which I have developed and tested modified protocols to improve the specificity of these tests for diagnosing GHD.

CHAPTER 1.2: NORMAL GROWTH AND GROWTH MONITORING

Growth is a sensitive indicator of health in childhood. The multifactorial influences on growth include psychological, systemic and nutritional factors[6, 14]. Hence the detection of abnormal growth prompts a more detailed systemic evaluation, requiring the physician to consider and rule out a long list of possible diagnoses[3, 15].

In this chapter, I will first provide an overview of normal childhood growth before outlining the clinical approach to children with abnormal growth patterns, including the diagnostic evaluation of GHD.

1.2.1 THE NORMAL PATTERN OF CHILDHOOD GROWTH

The observed pattern of normal childhood growth depends on the frequency of measurement. When measured every 6 to 12 months, a smooth and distinct growth trajectory is seen. This trajectory shows three different growth phases throughout childhood (Figure 1.2.1A). Weekly measurements unmask a seasonal variation in growth rate[16, 17] (Figure 1.2.1B), whereas daily measurements demonstrate growth bursts in a pattern of "saltation and stasis"[18, 19] (Figure 1.2.1C). An understanding of these patterns of observed growth is key to interpreting interval growth measurements in a particular child. Measurement error[20, 21], as well as diurnal variation[22, 23] in height, may introduce further challenges when small increments in childhood growth are being observed.

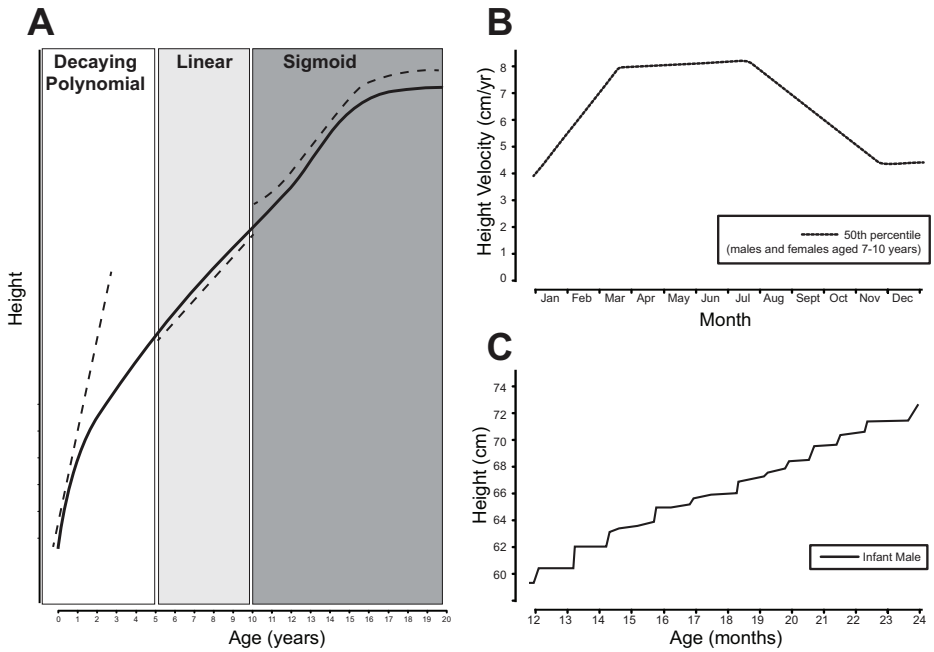

Figure 1.2.1: The observed patterns of linear growth seen when a child is measured six- to twelve-monthly (A), weekly (B) or daily (C).
Six- to twelve monthly measurements show distinct phases of constant growth throughout childhood (A). More frequent measurement unmasks seasonal variation, as demonstrated in 7-10 year old children measured three-monthly (adapted from Marshall[16]). Saltation and stasis of growth is seen if daily measurements are used, as shown in a male infant (adapted from Lampl[19])(C)

1.2.1.1 The Growth Chart

The earliest known longitudinal description of childhood growth was reported by George Louis Leclerc, who described Philibert Guneau de Montbeillard's measurements of his son every six months from his birth in 1759 until he was 18 years of age[24] (Figure 1.2.2). The concept of describing an individual's growth pattern relative to the population followed, with percentiles being proposed as a method of describing population anthropometric variation by Francis Galton in 1875[25]. Henry Bowditch was the first to design a chart of height and weight with percentiles in 1891[26], and his method has evolved into the growth charts that we use today[27]. Current growth reference standards utilise these charts to describe linear growth in reference populations. When used in paediatric practice, they allow for the identification of abnormal growth patterns in a child through comparison of an individual's growth measurements with population norms.

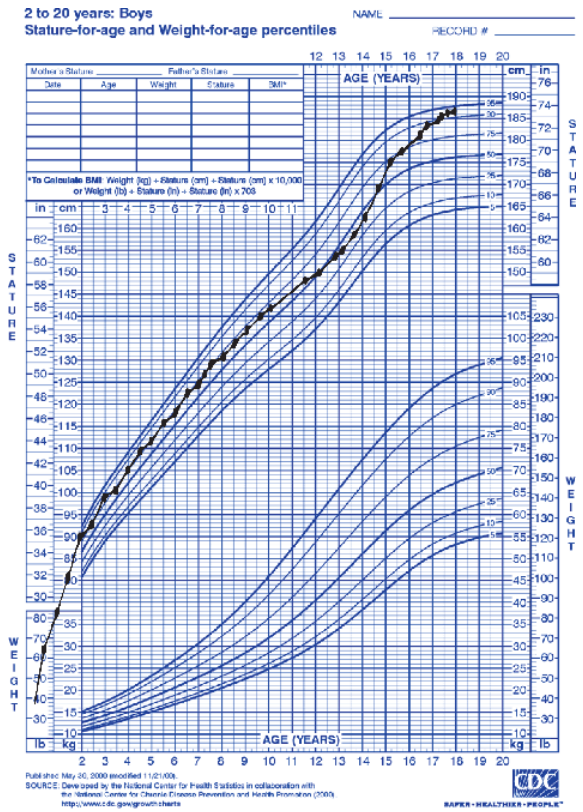

Figure 1.2.2: The linear growth of de Montbeillard's son (1759-1777), plotted on the modern Center for Disease Control Growth Chart. This was the first longitudinal description of the pattern shown in Figure 1.2.1A.

1.2.1.2 Linear Growth Pattern

When measured every six months to one year, growth follows a predictable pattern that can be described mathematically. This includes rapid but decelerating growth in infancy and early childhood, which slowly deviates from a straight line and has the appearance of a decaying polynomial. Growth is relatively constant and linear through middle childhood and this is followed by a sigmoid curve through adolescence (Figure 1.2.1)[28]. There are genetic and sex differences in the timing of these phases of growth[29], but the general patterns remain consistent.

1.2.1.3 Seasonal Growth

Leclerc also observed that there is seasonal variation[17, 30] in the rate of childhood growth. There are between three and six growth spurts each year, each lasting from 13 to 155 days[31]. Height velocity peaks in summer/autumn[17, 32], with

relatively slower growth in winter and spring[17, 23]. The difference in rate of growth between these seasons is as high as 30%[17]. This can give an appearance of fluctuating height velocity when followed closely throughout the year. Marshall demonstrated these observed patterns of growth velocity in children aged 7 to 9 years, and these data are shown in Figure 1B[16].

When considering longitudinal linear growth, the season of measurement can influence interpretation of growth pattern. Where a child's height is measured frequently, seasonal slowing of growth may be misattributed to pathology[33]. As will be discussed later, time of day and measurement error can also be of significance. This is particularly true when small increments in height are being observed.

1.2.1.4 Daily Growth

Daily height measurements also demonstrate that growth does not follow the linear pattern shown in Figure 1A. In fact, it occurs in bursts (saltation), interspersed with periods of stasis (Figure 1C). Lampl et al described this in 31 infants who were measured frequently over the course of 21 months. This group showed that infant growth only occurred during 5 to 10% of this time with bursts interspersed by periods of no growth lasting between 2 and 63 days[19].

1.2.1.5 Sources of Measurement Error

Accuracy of measurement is a significant challenge in describing and monitoring childhood growth. Where minor increments in growth are being detected, small errors may provide false concern or reassurance. The most common sources of error are related to equipment or human error.

People are taller in the morning than later in the day[22, 23], although the magnitude of this effect varies between studies. In a study of 53 children, Voss *et al* reported that 0.31 cm in height was lost between 09:00 and 11:00 am, with a further 0.2 cm being lost by 1 pm. Other studies have reported a decrease in height of up to 1.5 cm from morning to late afternoon[34, 35], with reversal of this

decrease after an afternoon nap[35]. It has been suggested that gentle upward traction on the child's mastoid processes would correct for this diurnal variation in height[23, 36], but this may just increase the measured height nonspecifically by approximately 3 mm[22].

Children under the age of two years should be measured supine by two observers, using a moveable footplate. The infant should be placed on a stable surface. Over the age of two years, a wall mounted stadiometer should be used[37]. Growth charts have been developed using this mode of measurement, and there is an approximately 0.7 cm difference between a supine and standing 2-year-old[37]. If a child under the age of two years is measured using a standing stadiometer, their recorded height percentile will be lower as the growth chart has been developed based upon supine measurements. After two years of age, the change in position of measurement results in a reduction in the expected height at each percentile. Juxtaposition of growth charts before and after two years of age demonstrates this effect, and is demonstrated in Figure 1.2.3[38-41].

Additional recommendations regarding technique are made in an effort to minimise inter-observer variation. Shoes should be removed and the child should stand against a wall with head, shoulders, buttocks and heels touching the wall. The child's head should be in the Frankfort plane, where the upper margin of the ear canal and lower margin of the orbits are on the same horizontal plane[37, 42]. Similar recommendations are made for the supine infant, but this can be difficult to achieve. To improve reliability, a minimum of two measurements within 4 mm of each other should be taken, and the average recorded[42].

Regular calibration of equipment is recommended to minimise this potential source of measurement error. The importance of this is highlighted by a study by Voss *et al* in 1990. This group evaluated 230 different measurement devices in active use in hospitals, schools, and primary care centres in the United Kingdom. They used the devices to measure a 100 cm rod and showed that measurements

ranged from 90 to 105.2 cm, and that microtoises tended to be fixed too low (resulting in an overestimation of the child's height)[43].

Despite closely following these recommendations, height measurement is often inaccurate. Inappropriate or damaged equipment[44], use of length instead of height, incorrect technique[20] and/or child movement may all contribute to unreliable measurements. Training and standardisation of equipment can improve accuracy and facilitate improved growth surveillance[21].

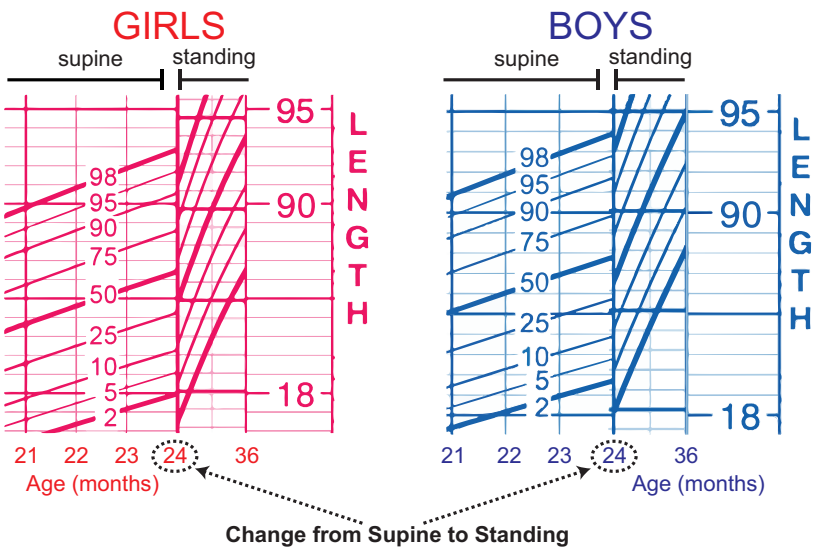

Figure 1.2.3: There is a reduction in expected height when the child's position changes from supine to standing. This figure demonstrates this reduction by juxtaposing the CDC supine 0-2 year and standing 2-20 year growth charts for boys and girls at 2 years (Adapted from CDC[38-41]).

1.2.2 The Medical Assessment of Faltering Growth

The differential diagnosis for faltering growth may include systemic illness, psychosocial deprivation, endocrine disorders (e.g. thyroid dysfunction, adrenal insufficiency) and familial growth patterns (e.g. constitutional delay in growth and puberty). Screening laboratory tests are indicated in all patients where an aetiology is not identified[45]. These tests are shown in Table 1.2.1.

Table 1.2.1: Recommended screening tests for children with unexplained short stature[45]

Test	Patients

Full Blood Count	All
Erythrocyte Sedimentation Rate	All
Creatinine	All
Electrolytes	All
Bicarbonate	All
Calcium	All
Phosphate	All
Alkaline Phosphatase	All
Albumin	All
Thyroid stimulating hormone, thyroxine	All
IGF-I	All
IGFBP-3	All
Celiac disease screen	All
Bone age x-ray	All
Karyotype	Boys with genital abnormalities All girls
Skeletal Survey	If body proportions suggest skeletal dysplasia, or height significantly below family target

Using this approach, up to 5% of children with unexplained short stature are identified as having an underlying cause[3]. However, in one study of 1373 patients, only 2% of patients with short stature had all of the recommended tests performed[15], and it has been suggested that this screening approach in the absence of symptoms may not be cost effective[15]. This study estimated the laboratory cost at over $100,000 for each new diagnosis identified by these tests alone.

1.2.3 Growth Hormone Deficiency

GH is a key mediator of childhood growth, and deficiency usually presents with short stature. An exception to this is in infancy, where GH plays a less prominent role in mediating growth. In infants with GHD, microphallus or hypoglycaemia are often the presenting clinical features[13, 46] (Chapter 1.4).

As will be briefly described here, and recur as a theme throughout this thesis, the diagnostic tests for GHD have poor specificity for disease. Many normally growing children without GHD will appear to have this condition if these tests

are relied upon to make a diagnosis[47-49], thus the tests should be considered "confirmatory" rather than "diagnostic". However, there are also conditions where patients with neurosecretory dysfunction (NSD) may pass a stimulation test but have GHD[50, 51] (see 1.2.4.3).

1.2.3.1 The cost of a GHD misdiagnosis

Between 30 and 50% of children treated with GH do not show a significant improvement in growth, indicating that the misdiagnosis of GHD is unfortunately common[52]. This difficulty in making the correct diagnosis is further highlighted by the variation in the reported prevalence of GHD from 1 in 2,000 to 1 in 30,000[53-55] children. This emphasises the need to improve the diagnostic tests for GHD, as unnecessary treatment with GH has significant costs for society, the family and, importantly, the child.

Recombinant GH is expensive and this cost is borne by society, either through taxes or health insurance costs. The estimated cost varies according to brand of medication used (Table 1.2.2), dosing schedule[56], IGF-I response to treatment[57] and weight. In the U.S., the cost is estimated at $15,000 per year for a 30 kg child, which can increase to over $50,000 in a pubertal adolescent[58, 59]. Consequently, GH generates almost $2 billion in annual sales revenue[60]. When used in the absence of GHD (i.e. idiopathic short stature), it has been argued that the cost of 4 cm additional growth achieved by treatment of one child could provide 200,000 vaccinations against measles for children in developing countries[61].

Table 1.2.2: Price Comparison for growth hormone products in Ireland in 2015 (per milligram)[62]. Note that the total annual cost does not include discarded growth hormone at the end of each vial.

Formulation	Cost per mg	Cost of 1 mg daily over one year
Genotropin (Pfizer)		
Go Quick 5.3 mg	€ 21.75	€ 7,938.75
Go Quick 12 mg	€ 26.53	€ 9,683.45
Miniquick	€ 28.65	€ 10,457.25
Norditropin (Novo Nordisk)		
SimpleXx 15 mg	€ 30.23	€ 11,033.95

SimpleXx 10 mg	€ 38.33	€ 13,990.45
SimpleXx 5 mg	€ 38.48	€ 14,045.20
Saizen (Merck Serono)		
20 mg	€ 30.51	€ 11,136.15
12 mg	€ 30.51	€ 11,136.15
8 mg	€ 30.51	€ 11,136.15
6 mg	€ 30.51	€ 11,136.15
Zomacton (Ferring)		
10 mg	€ 21.80	€ 7,957

GH is administered by daily subcutaneous injection, which may cause discomfort to the child[63]. Long-term surveillance studies have shown conflicting data regarding a potential increased risk of mortality, malignancy[64, 65] and stroke[66-69]. Other possible risks of treatment include intracranial hypertension, slipped capitulum of the femoral epiphysis[70], and exacerbation of scoliosis; although the latter two of these are considered to be secondary to increased linear growth. Although generally considered to have a good safety record, the Pediatric Endocrine Society and European Society for Paediatric Endocrinology have recommended continued surveillance of children and adults who have been exposed to GH treatment[71]. Given this background of concern for potential risks associated with treatment, avoiding treatment in children for whom there is minimal benefit would be preferred. However, the currently available diagnostic tools often do not accurately identify these children.

1.2.4 The Diagnostic Approach to Growth Hormone Deficiency

Current consensus guidelines for diagnosing GHD include the interpretation of height both absolutely and relative to the heights of the parents and siblings, height velocity, bone age, serum IGF-I and serum insulin-like growth factor binding protein 3 (IGFBP-3), and GHST[72]. Each of these tests has poor specificity for diagnosing GHD, making it extremely important to accurately measure children and to only investigate children with a clinical phenotype suggestive of GHD. The diagnostic steps are summarised in Figure 1.1.4.

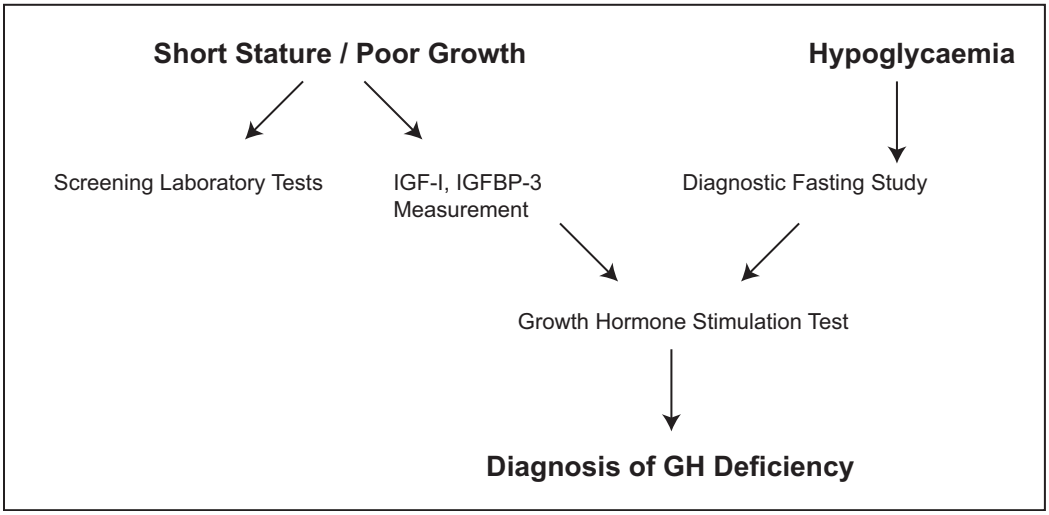

Figure 1.1.4: Current diagnostic approach to the child with suspected GHD

1.2.4.1 IGF-I Measurement

IGF-I is a 70 amino acid polypeptide[73] with structural similarities to insulin[73, 74]. The somatomedin hypothesis linking GH with hepatic IGF-I production and subsequent cellular growth was proposed in 1957[75], but has undergone numerous revisions. The current understanding of the complex relationship between GH, IGF-I and growth includes: GH-independent IGF-I mediated prenatal growth; GH-dependent postnatal growth mediated by interplay between hepatic IGF-I, IGFBP-3 and Acid Labile Subunit (ALS); and local autocrine and paracrine GH-dependent IGF-I production and action[76]. Despite circulating levels being comprised predominantly of hepatic IGF-I, local autocrine and paracrine action of IGF-I can maintain normal linear growth in the absence of hepatic IGF-I production[77]. Therefore, despite the established role of IGF-I measurement in the screening for GHD, it is possible that circulating IGF-I does not reflect IGF-I action at the growth plate.

The potential role of serum IGF-I measurement by radioimmunoassay to identify children with GHD was first proposed in 1977 by Furlanetto[78]. Copeland *et al* subsequently showed that IGF-I concentrations increase following GH administration[79]. Spontaneous GH secretion was then shown to be associated with serum IGF-I concentrations[80]. IGF-I and IGFBP-3 concentrations were then identified as a useful screen for GHD in children with short stature[2, 81], and

young adults with childhood onset GHD who were being evaluated for persistent GHD at completion of linear growth[1]. The reported sensitivity and specificity of low IGF-I concentrations for GHD are shown in Table 1.2.3. The gold standard test used for the diagnosis of GHD in most of these studies was the GHST, which, as will be described later in this chapter, has limitations. Regardless, the sensitivity of IGF-I measurement ranges from 34 to 86% and specificity from 22 to 97%.

Limitations and potential areas for optimising IGF-I measurement will be explored throughout this thesis. Interference by IGFBPs provides a significant challenge for assays and the potential for mass spectrometry to advance this field will be studied (Section 2). Nutritional status influences growth and IGF-I concentrations, and I will study this association with measures of body composition in infancy (Section 4). In addition to this, unique cellular aspects of IGF-I production and action will be discussed in Section 3.

1.2.4.2 IGFBP-3 Measurement

IGFBP-3 is a member of the IGF superfamily of proteins, which contains five other IGFBPs. It binds IGF-I with higher affinity than the IGF-I receptor, and prolongs IGF-I half life as well as modulating free bioactive IGF-I availability[82]. ALS and IGFBP-3 bind approximately 75% of circulating IGF-I in a ternary complex, with other IGFBPs contributing in smaller proportions to IGF-I binding[83, 84]. IGFBP-3 is expressed in numerous tissues, and differential local expression has been suggested to have an IGF-I independent role in colon[85], oesophageal[86, 87], prostate[88, 89], and breast[90] cancers.

Similar to IGF-I, IGFBP-3 concentrations reflect GH activity. Concentrations are elevated in acromegaly[91], and reduced in adults with GH receptor deficiency[92]. IGF-I treatment of adults with GH receptor deficiency does not normalise IGFBP-3 concentrations, demonstrating that IGFBP-3 production is dependent upon GH rather than IGF-I[92]. In addition to GH, other factors can increase IGFBP-3 concentration and these include parathyroid hormone, 1,25-

dihydroxyvitamin D[93], insulin[94], interleukin-1[95], and tumor necrosis factor alpha[96]. Factors such as androgens[97], estrogens[98], and glucocorticoids[99] can decrease IGFBP-3 concentrations.

IGFBP-3 measurement has an established role in the screening of short children with suspected GHD[8, 9], where low concentrations are suggestive of disease. However, the reported sensitivity and specificity of this approach varies from 22 to 97% and 60 to 100%, respectively[100] (Table 1.2.3).

1.2.4.3 Profiles of GH Secretion

GH is secreted in a pulsatile pattern, with increased amplitude of these pulses occurring during puberty. In childhood, these pulses are periodic and occur approximately every 200 minutes[101]. This pulsatile secretion represents a diagnostic challenge when trying to determine if a child has GHD.

The practice of performing 24-hour GH profiles has fallen from favour due to the labour-intensive nature of the test and limited additional information provided. Despite this, it has been suggested that some children with sufficient peak GH concentrations may have reduced spontaneous secretion[102], termed NSD. This may be more common in children following cranial irradiation, and has been reported in that context in children who have been treated for acute lymphoblastic leukaemia[50, 103, 104]. It is possible that NSD may be missed if the GHST is relied upon to make the diagnosis in GHD[105], although some studies have also shown no increase in the diagnostic yield by doing this 24-hour GH profile[106]. As previously mentioned, IGF-I concentrations can correlate with spontaneous GH secretion[80]. NSD may be considered in at-risk children with a suggestive clinical phenotype and low IGF-I concentrations, despite normal stimulated GH concentrations and normal nutritional status (Chapter 4.2).

Table 1.2.3: The sensitivity and specificity of IGF-I and IGFBP-3 measurement for GHD. Adapted from[100], MPHD=multiple pituitary hormone deficiency

Author	Population	GHD Diagnosis	IGF-1			IGFBP-3		
			Limit (Z)	Sens (%)	Spec (%)	Limit (Z)	Sens (%)	Spec (%)

Blum 1990[107]	132 patients with GHD (116 isolated GHD, 16 MPHD), mean age 11.2y (range 0.25-34.4).	Clinical diagnosis of GHD				-1.6	97	95
Hasegawa 1994[108]	59 children with GHD	Peak GH < 5 ng/ml				-1.6	92	69
Bussieres 2000[109]	43 prepubertal children with GHD (28 isolated GHD, 15 MPHD)	Peak GH <10 ng/ml	-1.6	72	95			
Nunez 1996[81]	104 children with GHD (aged 3-16 years)	Peak GH < 7 ng/ml	-1.7	69	76	-1.7	50	69
Boquete 2003[110]	34 children with GHD (23 isolated GHD, 11 MPHD)	Peak GH <7 ng/ml on 2 stimulation tests	-1.7	68	97	-1.8	90	60
Cianfarani 2002[111]	33 children with GHD (32 prepubertal)	Peak GH <10 ng/ml Abnormal MRI brain Positive growth response to GH Rx	-1.9	73	95	-1.9	30	98
Mitchell 1993[112]	318 patients with GHD (aged 0.9 to 25.4 years)	Peak GH < 5.2 ng/ml Height Velocity <-0.8 Z	-2	62	47	-0.5	61	68
Das 2003[113]	134 children with GHD (mean age 5.2y, range 0.1-16.9)	Clinical diagnosis of GHD	-2	86	100	-2	79	86
Lissett 2003[114]	244 children with childhood-onset GHD	Peak GH < 3 ng/ml	-2	86				
Rikken 1998[115]	63 children with GHD	Peak GH < 7.5 ng/ml	-2	65	78	-2	53	81
Tillmann 1997[116]	60 children with GHD (17 isolated GHD)	Height <-2 Z Height Velocity <-2 Z Delayed Bone Age >2y	-2	34	72	-2	22	92
Weinzimer 1999[117]	72 children with brain tumors and GHD	Height velocity < -2 Z Peak GH < 7 ng/ml	-2	73		-2	50	

1.2.4.4 GH Stimulation Testing

The GHST comprises the administration of a pharmacological stimulus for GH secretion and the subsequent serial measurement of GH concentrations. Various stimuli for GH secretion are used in the clinical evaluation of GH reserve, of which insulin induced hypoglycaemia is often considered to be the gold standard[118]. There are variations in protocols used for the timing of GH measurement during the insulin tolerance test (ITT), with most recommending measurement of GH concentrations at baseline and 30, 60 and 90 minutes following insulin administration. Other protocols recommend additional measurements within the first forty minutes following insulin administration[119-121]. Other commonly used pharmacological stimuli include clonidine, arginine, levodopa, and glucagon[122]. Similar to the ITT, protocols used for each of these stimuli include the serial measurement of GH after administration[119-121].

A peak GH concentration below an arbitrary threshold between 5 and 10 ng/ml is generally used to determine if a child has GHD, but this approach is problematic.

These thresholds were developed using GH measurements by polyclonal assays, and measurements using these may not be consistent with modern monoclonal assays[123]. There is even assay variability in GH measurements between monoclonal assays, so results may vary between hospitals depending on the specific assay used by the laboratory[124]. Another factor that may influence result interpretation is inter-individual variation in GH pharmacokinetics, resulting in measured serum GH concentrations not necessarily reflecting secreted GH[125].

When considering all of these issues, it is conceivable that these tests do not correlate well with response to GH treatment. The sensitivity of a stimulated GH peak concentration below 10 ng/ml for detecting prepubertal children who will have a height increase by 0.5 SDS within one year of GH treatment is 82%. However, specificity for these parameters is 24%[126]. Even lower peak GH concentrations also do not correlate well with growth response to treatment[126-128]. Almost a quarter of normally growing children without clinical features of GHD have a peak GH concentration of less than 7 ng/ml during GHST, and almost half will have a peak serum GH concentration of less than 10 ng/ml[47]. Although the sensitivity of these tests for GHD is favorable, many normal children without GHD will be characterised as having disease based on a low peak GH concentration on stimulation testing alone[47-49]. Thus, GHST result should be interpreted in the clinical context and should not be considered in isolation to diagnose GHD[72]. It should also be noted that these tests are not benign and mortality has been reported with the inappropriate management of hypoglycaemia following insulin or glucagon administration as part of a GHST[129].

Despite their poor reproducibility[130, 131] and the aforementioned challenges in interpreting results, the GHST remains central to diagnosing GHD[72]. Questions surrounding most aspects of GHSTs are debated amongst endocrinologists. These include when and how they should be performed, priming of peripubertal patients with sex steroids[132], assays used, and interpretation of GHTS results are issues that remain unresolved. Multiple surveys of practice have highlighted

variations in each of these areas[62, 133-135]. In 1995, only a third of endocrinologists believed that GH response to stimulation testing correlated with response to therapy[133], and more recent studies show that this hasn't changed. Only half of clinicians in 2010 reported that they would continue to use GHST if insurance providers did not insist on these prior to approving therapy[135].

1.2.4.5 Variation in Approach to diagnosing and treating GHD in Ireland

Given the aforementioned challenges in interpreting the diagnostic evaluation of the child with possible GHD, significant variations in clinical practice have been described through international questionnaire-based studies[122, 133, 136, 137]. I have performed a similar survey in Ireland, and shown variable approaches to each step of the diagnostic and therapeutic approaches to GHD (Appendix A). This includes differences in: number of failed GHSTs required to diagnose GHD; GH secretagogues used; sex steroid priming; diagnostic approach to GHD in neonates; criteria used to select GH brand used; criteria for stopping GH treatment; and retesting after completion of treatment[62].

1.2.5 SUMMARY

While monitoring childhood growth is a central component of general paediatric health surveillance, the interpretation of growth is complicated by measurement error, as well as daily, seasonal and annual growth variation. Medical causes of poor linear growth will be identified in 5% of cases by the recommended baseline laboratory screening. Evaluating for GHD is recommended in the subset of the remaining 95% with a suggestive clinical phenotype. IGF-I and IGFBP-3 measurement, as well as GHST play important roles in this evaluation but can often lead to the misdiagnosis of GHD. In this thesis, I will explore potential areas in which this diagnostic approach may be refined.

In the following chapters of this Section, I will describe novel interventions to improving the specificity of the GHST for diagnosing GHD in children with short stature (Chapter 1.3) and unexplained hypoglycemia (Chapter 1.4).

CHAPTER 1.3: IMPROVING THE SPECIFICITY OF THE GROWTH HORMONE STIMULATION TEST THROUGH SERIAL GROWTH HORMONE MEASUREMENT AFTER IV CATHETER PLACEMENT

Publication

Hawkes CP, Mavinkurve M, Fallon M, Grimberg A, Cody DC. Serial GH measurement after intravenous catheter placement alone can detect levels above stimulation test thresholds in children. J Clin Endocrinol Metab. 2015;100(11):4357-63. (Appendix B)

The GHST comprises a pharmacologic stimulus causing GH secretion, and serial serum GH being measured. In order to demonstrate GH sufficiency, one of these serum GH measurements should be greater than a predefined arbitrary threshold concentration. This is described in more detail in 1.2.4.

As early as 1968, Kaplan et al identified "excitement alone" as a stimulus for GH secretion that can complicate provocative testing[138]. In their study of 134 children, 53 of whom had hypopituitarism, 18 (22%) of the children without GHD had a fasting GH concentration of greater than 9 ng/ml prior to receiving insulin. The GH concentration prior to insulin administration was higher than the peak concentration after insulin induced hypoglycaemia in eight of these. Of note, none of the children with hypopituitarism had a stimulated peak GH concentration of greater than 2.2 ng/ml in this study.

The hypothesis was that intravenous line placement (IVP) stimulates GH secretion and may deplete GH reserve in some children undergoing subsequent ITT. Consequently, frequent measurement of GH concentrations after IVP may identify GH sufficient children who would be missed if ITT measurements alone were used.

1.3.1 AIM

The aim of this study was to determine if measurement of GH at baseline, 15 and 30 minutes after IVP would identify additional GH sufficient patients, not identified by subsequent ITT. The secondary aim was to determine if this was also relevant for cortisol response to ITT.

1.3.2 METHODS

The ITT protocol was modified at Our Lady's Children's Hospital Crumlin (OLCHC), Dublin, Ireland to include additional serum GH measurements in the thirty minutes following IVP but prior to insulin administration. All children undergoing ITT at OLCHC between January 2013 and December 2014 were included in this study. This study was approved by the Institutional Review Board of OLCHC.

1.3.2.1 Baseline Evaluation

IGF-I was measured by enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 XPi, Siemens Healthcare Diagnostics, Berlin, Germany). Z-scores were reported according to laboratory standards using chronological age and gender reference data. GH, cortisol and IGFBP-3 concentrations were also measured by enzyme-labeled chemiluminescent immunometric assays (Immulite 2000 XPi, Siemens Healthcare Diagnostics, Berlin, Germany). Bone age x-rays were performed and these were interpreted by paediatric radiologists using the standards of Greulich and Pyle[139].

1.3.2.2 Insulin Tolerance Test Protocol

According to protocol in this institution, all prepubertal patients with a recorded bone age over 10 years underwent sex steroid priming prior to insulin tolerance test. Variations in practice regarding sex steroid priming have been reported, and similar criteria to those used in this study for sex steroid priming are used in many centres internationally[136]. Sex steroid priming for males consisted of one 100 mg dose of intramuscular testosterone ten days prior to the test, and for

females consisted of oral ethinylestradiol 10 mcg daily for three days prior to the test.

Children fasted for 12 hours prior to undergoing ITT. Ethyl chloride topical analgesia spray was used prior to insertion of an intravenous catheter. If placement was unsuccessful, this procedure was repeated with the use of analgesia spray. One intravenous line was placed 30 minutes prior to insulin administration, and this was used for insulin administration as well as GH and cortisol sampling.

Serum GH and cortisol concentrations were measured at the time of IVP and again 15 and 30 minutes later (t= -30, -15, 0). Intravenous insulin (0.1 u/kg) was administered 30 minutes after IVP (t=0) and GH, cortisol and glucose concentrations were measured at 15, 25, 35, 60 and 90 minutes following insulin administration (Figure 1.3.1). A glucose concentration of less than 45 mg/dL after insulin administration was required for test to be included in this study.

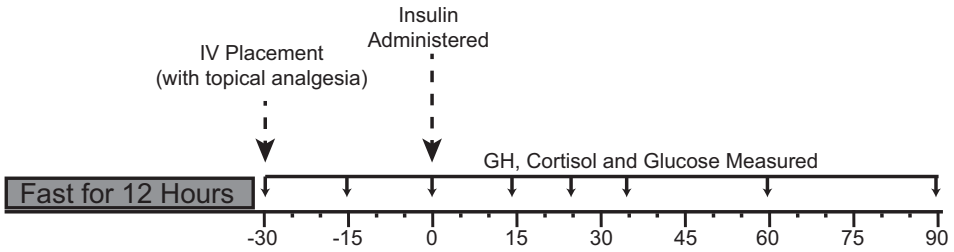

Figure 1.3.1: The protocol for insulin tolerance testing used in this study, with additional measurements prior to insulin administration marked.

1.3.2.3 Retrospective chart review procedure

Medical records were reviewed and weight, height, pubertal stage, parental heights, and comorbidities were recorded. IGF-I and IGFBP-3 concentrations, and bone age results were recorded. Puberty was defined as breast Tanner stage ≥ 2 in females or testicular volume ≥ 4 ml in males. Patient information was tabulated anonymously and maintained on a password-protected file on a secure server.

1.3.2.4 Statistical analysis

Patients were categorised according to the indication for ITT. Children undergoing initial evaluation of possible GHD were assigned to Group 1. Patients treated with GH who had completed linear growth and were undergoing ITT to determine if they had persistent GHD were assigned to Group 2. Peak stimulated GH concentrations of Groups 1 and 2 were analysed separately, but peak stimulated cortisol was analysed in both groups together. This distinction was made because many guidelines use different thresholds for stimulated peak GH concentrations in these two patient groups[9, 118, 140], whereas the threshold for peak stimulated cortisol concentrations is similar in both groups.

Height and weight z-scores at the time of stimulation test were generated using the World Health Organization standards[141], using STATA/SE version 12.0 (StataCorp, College Station, TX, USA). All data analyses were performed using SPSS 22.0 (IBM, NY, USA). Figures were generated using Prism 5.0 (GraphPad Software Inc, CA, USA) and Adobe Illustrator 16.0 (Adobe Systems Inc., California, USA).

1.3.3 Results

During the study period, 97 patients underwent 99 ITTs. Two tests were repeated, as the initial test did not induce hypoglycaemia below 45 mg/dL and were not included in the analysis. Of the 97 patients, 76 were evaluated for a possible new diagnosis of GHD (Group 1) and 21 had been treated for paediatric GHD, completed linear growth, and were evaluated at transition for adult GHD (Group 2). The demographics of the patients who underwent testing are shown in Table 1.3.1. In the children in Group 1, mean (SD) baseline IGF-I z-score was -1.2 (0.9) and bone age was delayed by 1.4 (1.9) years.

Table 1.3.1: Demographics of the patients undergoing insulin tolerance testing in this study. Continuous variables presented as mean (SD). *Children classified as having hypopituitarism have two or more anterior pituitary hormone deficiencies. CNS=Central Nervous System, SGA=Small for Gestational Age

	Group 1 (Initial Evaluation) (n=76)	**Group 2 (Transition) (n=21)**
Age (years)	10.9 (3.7)	17.1 (1.2)
Male (%)	58 (76.3%)	16 (76.2%)
Pubertal (%)	24 (31.6%)	21 (100%)
Comorbidity		
None	48 (63.2%)	11 (52.4%)
Hypopituitarism*	4 (5.3%)	1 (4.8%)
CNS mass or malignancy diagnosis	4 (5.3%)	5 (23.8%)
SGA	3 (3.9%)	0
Genetic Syndrome or Dysmorphism	8 (10.5%)	0
Other Dx (not associated with GHD)	7 (9.2%)	4 (19%)
Height z-score	-2.46 (0.96)	-0.73 (0.88)
Weight z-score	-1.75 (1.37)	-0.08 (1.27)
Midparental Height z-score	-0.29 (0.78)	0.22 (0.73)
Midparental Height z-score minus Height z-score	2.18 (0.85)	0.22 (0.73)

Serial GH measurement following IVP and prior to insulin administration increased the number of children passing the stimulation test from 8/76 (10.5%) to 19/76 (25%) if a threshold of 7 ng/ml were used (Table 1.3.2). Many patients in Group 1 had peak stimulated GH concentrations above thresholds of 3 ng/ml, 5 ng/ml and 7 ng/ml fifteen minutes after IVP (Figure 1.3.2), demonstrating that this measurement might identify additional children with sufficient GH secretion.

Of the 11 children with peak GH concentrations ≥ 7 ng/ml after IVP only, all were being investigated for isolated GHD and none had a coexisting risk factor for deficiency such as CNS malignancy or hypopituitarism. Three of these children had peak GH concentrations at the time of IVP, five peaked fifteen minutes later and five were 30 minutes later (Figure 1.3.3). Only one of these children had peak GH concentrations ≥ 7 ng/ml at more than one of these time points. This group had a mean (SD) age of 11.6 (3.9) years, IGF-I z-score of -1.3 (1), IGFBP-3 z-score of 0.8 (1.3), midparental height z-score of -0.04 (0.8), height z-score of -2.4 (0.9), weight z-score of -2.1 (0.8), bone age delay of 0.6 (2.1) years, and peak cortisol on ITT of 20.6 (3.5) mcg/dL. These were not

significantly different than the age (10.2 (3.8), p=0.4), IGF-I z-score (-1.2 (0.9), p=0.6), IGFBP-3 z-score (1 (1), p=0.9), midparental height z-score (-0.3 (0.8), p=0.3), height z-score (-2.5 (1), p=0.9), weight z-score (-1.7 (1.4), p=0.6), bone age delay (1.6 (1.9) years, p=0.2) or peak cortisol on ITT (18.7 (5.7) mcg/dL, p=0.2) of the other children in Group 1.

Table 1.3.2: The number of tests with peak stimulated growth hormone concentrations greater than 3 ng/ml, 5 ng/ml and 7 ng/ml following intravenous line placement, insulin administration, or both.

	Group 1 (Initial Evaluation) (n=76)	Group 2 (Transition) (n=21)	All (n=97)
Peak GH ≥ 3 ng/ml			
None	30	11	41
Intravenous line Only	13	1	14
Insulin induced hypoglycaemia Only	18	7	25
Both	15	2	17
Peak GH ≥ 5 ng/ml			
None	48	12	60
Intravenous line Only	10	3	13
Insulin induced hypoglycaemia Only	11	6	17
Both	7	0	7
Peak GH ≥ 7 ng/ml			
None	57	13	70
Intravenous line Only	11	2	13
Insulin induced hypoglycaemia Only	5	6	11
Both	3	0	3
Peak Cortisol ≥ 18 mcg/dL			
None	31	9	40
Intravenous line Only	2	2	4
Insulin induced hypoglycaemia Only	33	7	40
Both	10	3	13

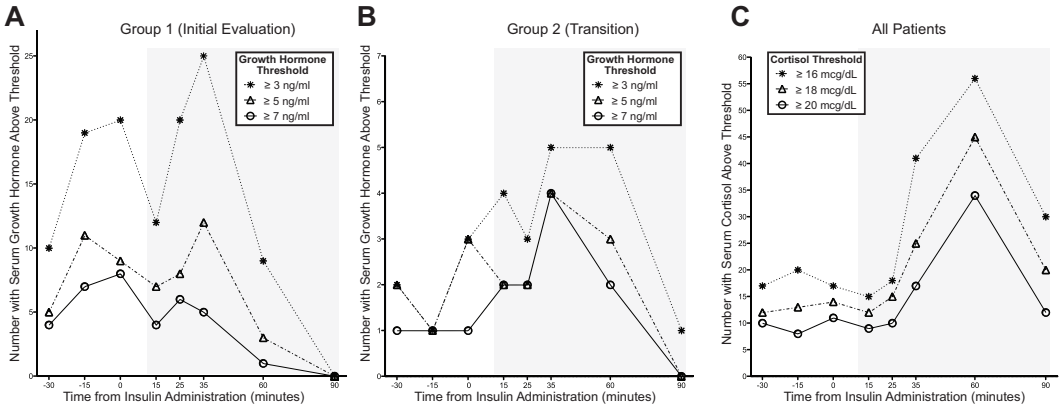

Figure 1.3.2: The number of patients with growth hormone (A, B) or cortisol concentrations above the defined thresholds in the 30 minutes following intravenous line placement (Time -30 to 0) and following insulin administration (Time 15 to 90 minutes, shown with a grey background). The intravenous line was placed at time -30 minutes, and insulin was administered at time 0 minutes.

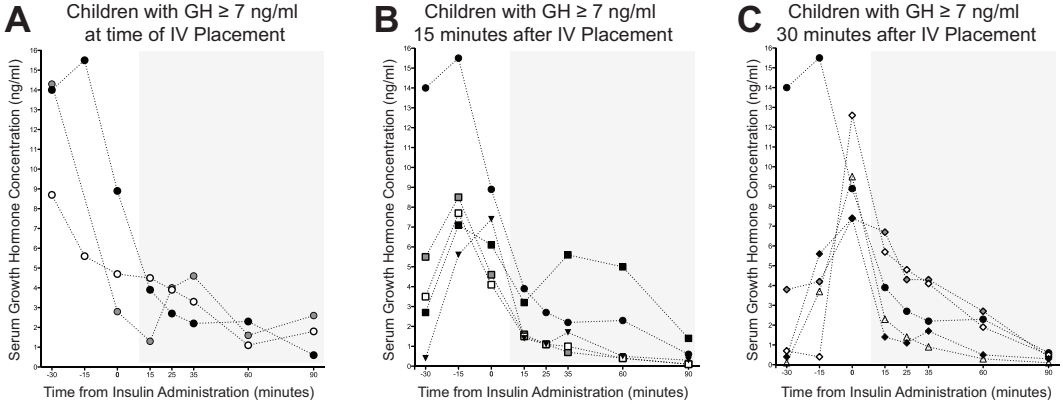

Figure 1.3.3: The serum growth hormone concentration profiles in children in Group 1 whose peak growth hormone concentration was ≥ 7 ng/ml at 0 (A), 15 (B) and 30 (C) minutes from intravenous line insertion without a subsequent peak ≥ 7 ng/ml after insulin administration. Each symbol and line represents the growth hormone profile in one patient. Note that one patient had growth hormone concentrations ≥ 7 ng/ml at each of these time points and is represented in each diagram.

Serial measurement of GH concentrations after IVP in Group 2 had a less significant effect on the number of patients reaching a predefined threshold GH concentration. The number of patients passing the test increased from 9/21 (42.9%) to 10/21 (47.6%) if a threshold of 3 ng/ml was used, or from 6/21 (28.6%) to 8/21 (38.1%) if a threshold of 7 ng/ml was used (Figure 1.3.2, Table 1.3.2). The total number of patients in both Groups 1 and 2 combined with a peak stimulated cortisol concentration of ≥18 mcg/dL increased from 57/97 (58.8%) to 61/97 (62.9%) with serial measurement before insulin administration. This represents an increase in specificity of 6.5%.

Peak GH concentration after IVP did not correlate closely with peak GH after ITT (Group 1 R^2 = 0.05, Group 2 R^2 = 0.002). In contrast the peak cortisol concentration prior to insulin administration was more closely related to the peak cortisol concentration during ITT (Group 1 R^2 = 0.26, Group 2 R^2 = 0.41) (Figure 1.3.4).

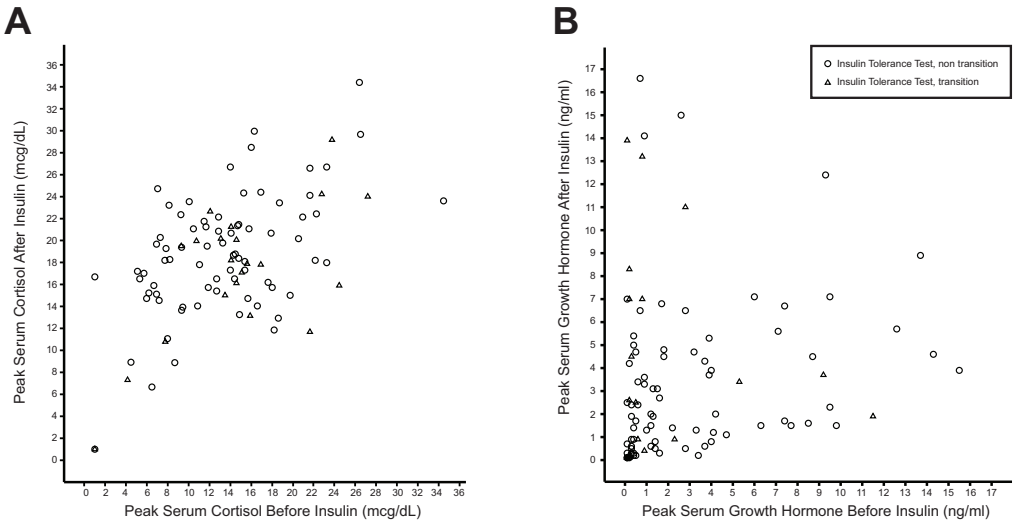

Figure 1.3.4: Peak cortisol (A) or growth hormone (B) in the 30 minutes prior to insulin administration plotted against the peak after administration of insulin.

1.3.4 DISCUSSION

We have demonstrated that many children undergoing evaluation for GHD can have a peak GH concentration greater than 7 ng/ml within 30 minutes of IVP, even without pharmacologic stimulation. The subsequent insulin induced hypoglycaemia during ITT does not always replicate this peak stimulated GH concentration. As a result, serial measurement of GH concentration following IVP may identify additional children with GH sufficiency who would not have been detected by ITT alone. Similar additional serial measurements of cortisol do not add significantly to the identification of children with adrenal sufficiency using the ITT.

The hospital environment and IVP may represent stressors to the child undergoing GHST, and stress may be a stimulus for GH secretion[142]. Some

authors have recommended that patients be admitted the night before a GHST, in order to acclimatise to the environment and to optimise the utility of the test[143]. However, this is often impractical due to limited hospital resources. Most centres perform these tests in the outpatient setting, often completing two separate tests on the same day[62, 133]. We have shown that frequent GH measurement after IVP should be considered in the context of GHSTs being performed as a day procedure.

Thirteen children (11/76 in group 1 and 2/21 in group 2) in this study had a peak GH concentration greater than 7 ng/ml following IVP without a subsequent response of similar magnitude to insulin-induced hypoglycaemia. Although we have performed this analysis only in children undergoing ITT, it is likely that this phenomenon is seen with IVP prior to the administration of alternative GH secretagogues. It is not clear if a stimulated GH surge prior to ITT will reduce the likelihood of subsequent response to ITT and there are children in this study with peak GH concentrations above the thresholds following both sequential stimuli. However, it is possible that a GH peak can have a negative effect on subsequent stimuli. In adult volunteers, sequential stimuli of GH secretion can result in an attenuated response to the second stimulus, although this is less pronounced when insulin induced hypoglycaemia is the second stimulus[142]. Similarly, the GH response to frequent bouts of heavy exercise is attenuated when the duration between these is reduced to less than one hour[144]. Possible mechanisms for the reduction in GH response to a second stimulus include depletion of immediately releasable pituitary GH by the first stimulus, or an effect of GH autoregulation. GH inhibits GHRH production through direct negative feedback and indirectly via the production of somatostatin and IGF-I[145], both of which exert negative feedback on hypothalamic GHRH production and pituitary GH exocytosis[146].

While a large proportion of children categorised as having GH insufficiency on GHST will not respond to GH treatment, there are also children who have sufficient GH secretion and respond well to GH treatment. It seems likely that

the children who responded to IVP with a GH level greater than 7 ng/ml are truly GH sufficient, but this assumption cannot be verified because there is no gold standard test for GHD. In this centre, a stimulated GH threshold of greater than 7 ng/ml was used to determine whether or not GH was prescribed. Thus, we cannot determine whether additional GH measurement reduced the sensitivity of the provocative test for GH responsive short stature. We also note that 37% of the children in this study did not have a peak cortisol concentration greater than 18 mcg/dL. This may be due to the heterogenous group of patients included, many of whom had hypopituitarism or a CNS malignancy. In addition, we note that many children without adrenal insufficiency will have a peak stimulated cortisol of less than 18 mcg/dL on ITT[147].

The additional benefit of serial GH measurement following IVP shown in this study may be ameliorated by variations in practice elsewhere. As previously mentioned, the specificity of a single GHST for GHD is poor and many centres perform two tests to improve this specificity. The use of a second stimulation test in the patients described in this study may have detected more of the children with peak GH concentrations above the threshold following IVP. At the time of this study, there was no consensus regarding sex steroid priming prior to GHST in prepubertal children but the practice of using this for children with bone ages greater than 10 years is consistent with many centres[136] and the recent Pediatric Endocrine Society guidelines[72]. Sex steroid priming in younger prepubertal children may increase the specificity of the GHST for GHD[148], and it is possible that serial GH measurement following IVP would provide less additional benefit if sex steroid priming was used in all prepubertal children undergoing GHST. Thus, the results of this study should be interpreted in the context of serial measurements after IVP with a single stimulation test in a centre where sex steroid priming is only used in limited circumstances. Specifically, we report that serial GH measurement after IVP detected 13 children (13.4%) with a peak stimulated GH concentration of greater than 7 ng/ml, but it is not known if these children would have otherwise been detected in combination with a second GHST or sex steroid priming.

In conclusion, we advise caution when performing GHSTs, as delays in GH measurement may increase the number of patients misclassified as having insufficient GH secretion. If the GH secretagogue or stimulus is administered immediately following IVP, it is important to ensure that there are frequent additional measurements in the first 30 minutes following placement and administration of the GH provocative test substance. Also, if there is any delay in administering the intravenous stimulus following IVP, the frequent sampling of GH in the first thirty minutes following IVP should be performed.

1.3.5 CHAPTER CONCLUSION

The poor specificity of the GHST is well established, and have caused many endocrinologists to question whether or not these tests should even be performed[149-151]. Any modification of this test that improves specificity is welcome, and will facilitate the diagnostic evaluation of children with suspected GHD. This study has been instrumental in the modification of the GHSTs at OLCHC and Children's University Hospital Temple Street. All institutions in Dublin now measure serial GH concentrations after placing IV lines in children undergoing GHST.

CHAPTER 1.4: EVALUATING CHILDREN WITH HYPOGLYCAEMIA FOR GROWTH HORMONE DEFICIENCY

Publications

Hawkes CP, Stanley CA. Pathophysiology of neonatal hypoglycemia. In: Polin RA, Fox WW, Abman SH, editors. Fetal and Neonatal Physiology: Expert Consult - Online and Print (In Press). 5 ed: Elsevier/Saunders; 2016. p. 1550-60 (Appendix C).

Hawkes CP, Grimberg A, Dzata VE, De Leon DD. Adding glucagon-stimulated GH testing to the diagnostic fast increases the detection of GH-sufficient children. Horm Res Paediatr. 2016;85(4):265-72 (Appendix D).

Presentation

Hawkes CP, Dzata VE, Grimberg A, De Leon DD. Integrating growth hormone testing with hypoglycaemia investigation. American Pediatric Society / Society for Pediatric Research, May 2014; Vancouver (Appendix E).

GH does not play a significant role in regulating prenatal growth, and length at birth in infants with congenital GHD is within the normal range. This is not the case for IGF-I, where infants with IGF-I resistance or deficiency can present with prenatal growth restriction[152, 153]. Thus, identifying the infant with congenital GHD is a challenge.

The two largest studies of infants with GHD have characterised the clinical phenotype[13, 46]. Bell et al included 169 infants with GHD who presented with hypoglycaemia in their study, over 90% of whom also had additional pituitary deficiencies and abnormal pituitary MRI evaluations[13]. Herber et al described 29 children with GHD and demonstrated that hypoglycaemia is more common in those presenting before 6 months of age, than after the first 6 months of life[46]. As

the child becomes older, persistent impaired fasting tolerance is seen but hypoglycaemia becomes less problematic[154].

Given that neonatal hypoglycaemia may be the only presenting sign of children with congenital GHD, the diagnostic evaluation of infants with unexplained hypoglycaemia should include consideration of this potential underlying diagnosis. The diagnostic tests for GHD in infants are even more challenging than in older children. IGF-I measurement in this age group is of limited utility with current assays (Chapter 2.2)[124]. GH measurement during hypoglycaemia has been suggested, and even erroneously described by Bell as "a quick and definitive diagnostic tool"[13]. The specificity of such an approach has subsequently been shown as close to 30%[12].

Hypoglycaemia in childhood can have numerous causes, and can even represent normal physiology in the first few days of life. In this chapter, I will provide an overview of the physiologic mechanisms for maintaining normoglycemia, highlighting the broad differential diagnoses for hypoglycaemia, which include GHD. I will then describe a study in which I have modified the local diagnostic evaluation of children with unexplained hypoglycaemia and suspected GHD to improve the specificity for GHD.

1.4.1 Overview of Glucose Homeostasis

Clinical hypoglycaemia is defined as a plasma glucose concentration low enough to cause signs or symptoms of impaired brain function. Recognition may be difficult when the patient cannot communicate symptoms (e.g., infants and neonates), but identifying hypoglycaemia is critical in the prevention of complications. The brain has a very high rate of metabolism and depends on a constant supply of glucose. Because the brain has little or no stores of glycogen, interruption of glucose delivery can have devastating consequences, including seizures and permanent brain injury[155-157].

As shown in Figure 1.4.1, the first stage of defence against hypoglycaemia is suppression of insulin secretion by pancreatic islets as plasma glucose concentrations fall below 80 mg/dL[158-162]. The second stage is secretion of counter-regulatory hormones to stimulate glucose release from liver glycogen stores when plasma glucose concentrations fall to ~65 mg/dL (glucagon release from pancreatic islets and sympathetic discharge as reflected by a rise in plasma epinephrine). Plasma cortisol and GH concentrations also rise as glucose falls to ~60 mg/dL[163, 164]. While increases in these hormones do not affect glucose levels acutely, they are required for long-term glucose homeostasis. The third stage of response to hypoglycaemia is the impairment of brain function itself at a glucose threshold of ~50 mg/dL. The glucose thresholds shown in Fig 1.4.1 come primarily from studies in adults, but they have been shown to also apply to children[164, 165]. In addition, observations of responses to hypoglycaemia in infants and children with various hypoglycaemia disorders suggest that glucose thresholds are essentially the same across all ages[166, 167].

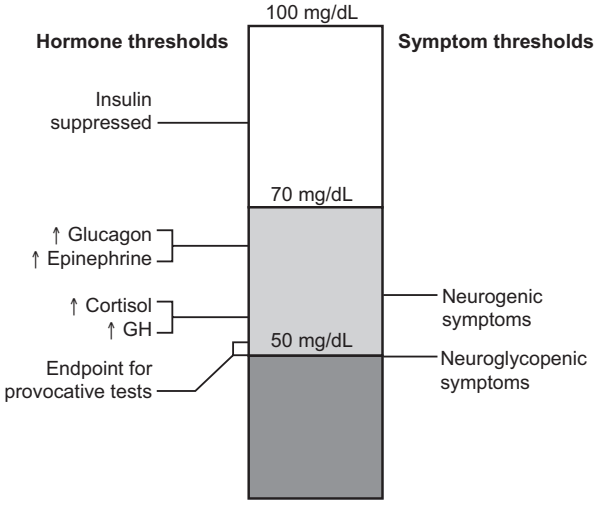

Figure 1.4.1: Glucose thresholds for neuroendocrine and neuroglycopenic responses to hypoglycaemia.
This demonstrates the progressive physiological responses to falling plasma glucose concentrations, and the thresholds for symptoms of hypoglycaemia (Adapted from Cryer et al[158, 168, 169]). The threshold used for ending provocative fasting studies and for performing laboratory evaluation of the fasting fuel response to hypoglycaemia is 50 mg/dL, demonstrated by the dashed line.

The importance of correctly detecting, diagnosing and treating infants with disorders of hypoglycaemia is emphasised by longitudinal follow up studies

demonstrating adverse complications. Kaser et al[170] used data from a statewide fourth-grade school exam and found that children who had a single plasma glucose level below 40 mg/dL during the neonatal period had a 50% lower odds ratio for proficiency for both literacy (0.43, 95% CI 0.28-0.67) and mathematics (0.51, 95% CI 0.34-0.78). Those with a single plasma glucose level below 35 mg/dL had even lower odds ratios for proficiency, (0.49, 95%CI 0.28-0.83) and (0.49, 95%CI 0.29-0.82), respectively. Even infants with a single glucose below a cutoff as high as 45 mg/dL had a reduced odds ratio for proficiency in literacy (0.62, 95% CI 0.45-0.85), although not mathematics (0.78, 95% CI 0.57-1.08). Koivisto et al[171] followed neurodevelopmental outcomes in 151 newborns with blood glucose concentrations below 30 mg/dL. They found that only 38% of infants who had a hypoglycaemic seizure had normal development at 4 years, compared with 80% of asymptomatic infants with glucose concentrations in this range.

Thus, in newborns, the brain is sensitive to hypoglycaemia and early diagnosis of the causative aetiology and initiation of the correct treatment of infants with disorders of hypoglycaemia is of great importance. Incorrectly diagnosing a child with GHD in this context, for example, may result in treatment with GH but risk persistent recurrent hypoglycaemia.

1.4.2 Improving the Specificity of the Diagnostic Fast for Growth Hormone Deficiency

GH plays an important role in the regulation of substrate use in the fasting state. Prolonged fasting results in an increase in frequency and amplitude of GH bursts[172] and GH secretion increases as blood glucose falls below 60 mg/dL[173, 174]. GH promotes lipolysis[175] and reduces utilisation of protein[176-178] and glucose[179]. It also induces insulin resistance through direct downstream effects on insulin signaling[180, 181], as well as indirectly by increasing non-esterefied free fatty acid concentrations[175]. Consequently, hypoglycaemia can be a presenting feature of GHD[13, 154, 182].

The standard approach to determining the aetiology of hypoglycaemia includes the measurement of the fuel and hormonal responses in the "critical" lab sample drawn during hypoglycaemia[183, 184]. Where possible, these serum markers are measured during opportunistic hypoglycaemia, but a structured diagnostic fasting study may be required. Although GH concentrations increase as blood glucose concentration falls (Figure 1.4.1), low GH concentrations during hypoglycaemia are commonly seen at the time of drawing the critical sample even in the absence of GHD[12, 185-187].

The evaluation of GHD as a potential cause of hypoglycaemia is complicated by the lack of a reliable and specific gold standard test for this condition, as also discussed in Chapters 1.2 and 1.3. Glucagon is used clinically to assess the inappropriate availability of glucose stores (glycogen) during hypoglycaemia. In a child with hypoglycaemia, an inappropriate rise in glucose concentration following glucagon administration may be consistent with hyperinsulinism[183, 188] or hypopituitarism (in the newborn period)[189]. Glucagon is also a GH secretagogue, and serial measurement of GH concentration following glucagon administration is used as one of the GHSTs of GH sufficiency in children with suspected GHD[190]. When intramuscular glucagon is used to assess GH secretion, peak GH concentrations are generally seen between 90 and 120 minutes following administration[191, 192]. Although glucagon is administered at the end of many fasting studies to evaluate glycogen stores, the routine serial measurement of GH following this glucagon administration is not performed as part of this fasting study.

1.4.2.1 Aim

The aim of this study was to determine if, in the setting of a diagnostic evaluation of a child with unexplained hypoglycaemia, serial measurement of GH concentrations following glucagon administration would improve the positive predictive value of the diagnostic fasting study for GHD.

1.4.2.2 METHODS

This study was performed at The Children's Hospital of Philadelphia (CHOP). All children presenting to this hospital between July 2012 and March 2015 were eligible for inclusion. Only children with unexplained hypoglycemia for whom there was a clinical concern for GHD as a possible diagnosis had the modified diagnostic fasting protocol performed (described below). This study was approved by the Institutional Review Board at CHOP.

Protocol

Given the poor specificity of GH measurement during hypoglycaemia for GHD[12], a clinical protocol for incorporating serial GH measurement after glucagon administration during hypoglycaemia was developed. The only additional intervention in this clinical protocol beyond the standard diagnostic fasting study was the serial measurement of GH following glucagon administration.

At the CHOP, the evaluation of children with unexplained hypoglycaemia includes a diagnostic fasting study[188, 193, 194]. The maximum fasting time used in this evaluation is age dependent. Children aged less than 1 month fast for up to 18 hours, between 1 and 12 months for up to 24 hours, and over 1 year for up to 36 hours. The monitoring protocol depends on the clinical scenario, but generally includes blood glucose monitoring during the fast using a bedside glucometer (Nova StatStrip point-of-care glucose monitor, Nova Biomedical Corporation, Waltham, MA, USA) every 3 hours until blood glucose is less than 70 mg/dL, hourly until less than 60 mg/dL and every 30 minutes until less than 50 mg/dL. Betahydroxybutyrate is also measured at the bedside every 3 hours using a handheld meter (PrecisionXtra, Abbott Laboratories). The study is ended when a confirmatory glucose concentration of less than 50 mg/dL is recorded, if beta-hydroxybutyrate concentration exceeds 2.5 mmol/L, or if the maximum pre-determined fasting time is reached.

A full diagnostic blood draw is taken at the end of the fast. This includes measurement of glucose, free fatty acids, beta hydroxybutyrate, insulin, ammonia, lactate, basal metabolic profile, acylcarnitine, c-peptide, carnitine, IGFBP-1, cortisol and GH. At the end of the diagnostic fast, glucagon is administered intravenously at a dose of 1 mg for all patients, and glucose is measured every 10 minutes by bedside glucose meter. Dextrose is administered if blood glucose does not rise by at least 20 mg/dL within 20 minutes. However, if glucose concentration rises by at least 20 mg/dL, then blood glucose checks are continued for a further 20 minutes before the fast is ended. If at any point during the glucagon test the child is unwell, dextrose is administered. The administration of dextrose at the end of the fast consists of a 2 ml/kg bolus of 10% dextrose and the child is provided a meal containing 40g of carbohydrates.

In addition to the standard protocol, additional GH concentrations were measured in patients in whom there was clinical suspicion of GHD. GH concentrations were measured at 30, 60, 90, 120, 150, 180 and 210 minutes following glucagon administration (Figure 1). Where there were clinical limitations to the blood volume that could be drawn, the 90 and 120 minute specimens were prioritised.

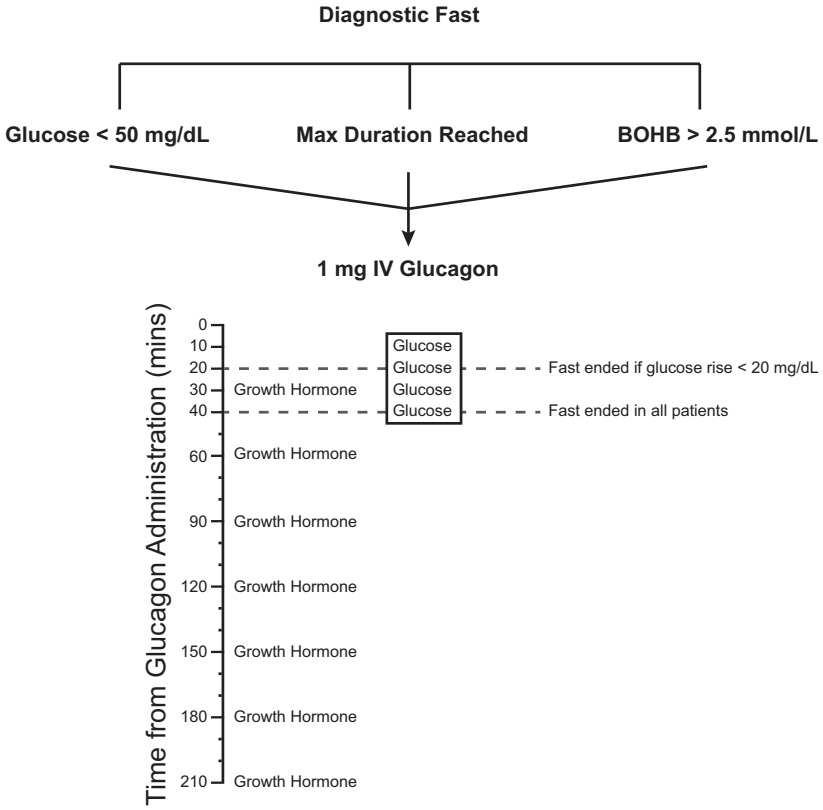

Figure 1.4.2: Protocol for additional growth hormone measurements after glucagon administration, in the context of a diagnostic fasting study

Laboratory Measurement

GH was measured by a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000, Siemens, Berlin, Germany), plasma glucose by an oxidase colorimetric reaction (Vitros 5600, Ortho Clinical Diagnostics, New Jersey, USA) and betahydroxybutyrate by a D-3 hydroxybutyrate dehydrogenase colorimetric reaction (Vitros 5600, Ortho Clinical Diagnostics, New Jersey, USA). IGF-I was measured by radioimmunoassay after acid ethanol extraction (Esoterix Laboratories, Texas, USA) and IGFBP-3 was measured by radioimmunoassay (Esoterix Laboratories, Texas, USA). Where relevant, assay-specific z-scores for age and gender were reported.

Statistical Analysis

The threshold GH concentration considered to represent GH sufficiency varies between centres and generally ranges from 5 to 10 ng/ml[137, 195]. In our centre, we use 7 ng/ml as the threshold for GH sufficiency. In this study, we also describe

overall data if thresholds of 5 ng/ml or 10 ng/ml were used, as varying GH concentrations are considered to demonstrate sufficiency in different centres.

Height and weight z-scores at the time of stimulation test were generated using the World Health Organization standards[141]. Unless otherwise stated, continuous variables were presented as median (IQR). All data analyses were performed using SPSS 22.0 (IBM, NY, USA). Figures were generated using Prism 5.0 (GraphPad Software Inc, CA, USA) and Adobe Illustrator 16.0 (Adobe Systems Inc., California, USA).

1.4.2.3 RESULTS

Twenty-nine patients were enrolled in the study. Median (IQR) height z-score was -2.3 (-3.3, -1). Of these patients, six had a final diagnosis of GHD and were treated with GH. The remaining patients had a diagnosis of hyperinsulinism (n=9), ketotic hypoglycaemia (n=13) or mitochondrial disorder (n=1). Out of all children included in this study, 4 (14%), 3 (10%) and 1 (3%) had GH concentrations above thresholds of 5, 7 and 10 ng/ml respectively at the end of the diagnostic fast. The additional GH measurement after glucagon administration identified 24 (86%), 19 (66%) and 15 (52%) children with GH concentrations exceeding these thresholds, who would have been missed if baseline GH concentration alone was used. The demographics of the patients included in this study are shown in Table 1.4.1.

Table 1.4.1: Demographic data, diagnoses, critical sample measurements and serial growth hormone concentrations following glucagon administration are shown.
Note that n represents the number of datapoints available for GH measurement at each timepoint. All data are presented as median (IQR).

Demographics	
Male, n (%)	16 (55%)
Age, years	1.8 (0.7, 3.4)
Height z-score	-2.3 (-3.3, -1)
Weight z-score	-1.2 (-2.3, -1)
Duration of fast, hours	15 (9.8, 20)
Final Diagnosis:	
Hyperinsulinism Ketotic Hypoglycaemia Mitochondrial Disorder Growth Hormone Deficiency	9 (31%) 13 (45%) 1 (4%) 6 (21%)
Concentrations at end of fast	
Glucose, mg/dL	45 (42, 51.5)
Cortisol, mcg/dL	16 (10.3, 20.5)
Betahydroxybutyrate, mmol/L	2.4 (1.5, 2.75)
GH measurements (ng/ml)	
Baseline (n=29)	2.6 (1, 3.5)
30 minutes (n=16)	2.5 (1.6, 8.2)
60 minutes (n=18)	4.9 (2.4, 10.7)
90 minutes (n=27)	5.6 (4.2, 12.9)
120 minutes (n=27)	4.4 (2.5, 10.2)
150 minutes (n=21)	5.1 (2.2, 11)
180 minutes (n=7)	3.2 (1.2, 11.6)
210 minutes (n=3)	3.6 (2, 4.9)
Number of subjects who exceeded threshold on testing	
GH > 5 ng/ml at Baseline	4 (14%)
GH > 5 ng/ml after Serial Measurements	25 (86%)
GH > 7 ng/ml at Baseline	3 (10%)
GH > 7 ng/ml after Serial Measurements	19 (66%)
GH > 10 ng/ml at Baseline	1 (3%)
GH > 10 ng/ml after Serial Measurements	15 (52%)

Of the 29 patients in this series, only three (10%) had GH concentrations above 7 ng/ml at the end of the fasting study and all three of these children also had GH measurements above this threshold again on serial testing. The percentage of samples with GH concentrations above 5 ng/ml, 7 ng/ml and 10 ng/ml at each timepoint is shown in Figure 1.5.3.

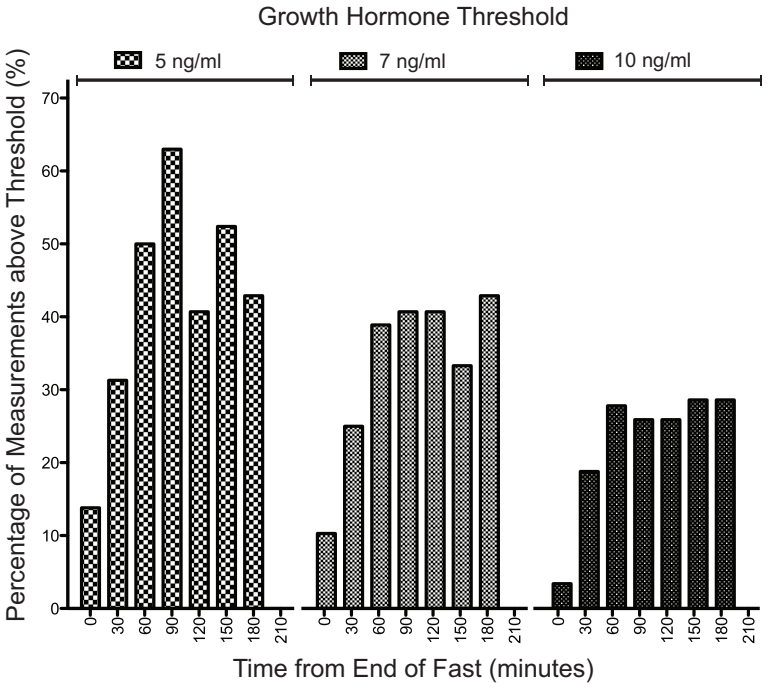

Figure 1.5.3: The percentage of children with growth hormone concentrations greater than or equal to thresholds of 5, 7 or 10 ng/ml at the time of glucagon administration at the end of the diagnostic fasting study (n=29), or 30 (n=16), 60 (n=18), 90 (n=27), 120 (n=27), 150 (n=21), 180 (n=7) or 210 (n=3) minutes later.

Of the 26 (90%) patients with GH concentrations less than 7 ng/ml during hypoglycaemia, 10 (34%) also had peak GH concentrations below this threshold on serial measurement after glucagon administration. Of these ten children without GH concentrations above 7 ng/ml, nine underwent additional GH stimulation testing using arginine and clonidine. Six were diagnosed with GHD and treated with GH. Two children were diagnosed with hyperinsulism and one with ketotic hypoglycaemia.

Characteristics and diagnostic evaluation of the ten patients with suboptimal peak GH concentrations are shown in Table 1.5.2. Additional diagnostic information on cases 7 and 8 from this series is provided, as the diagnosis may be unclear from the data presented in this table. Although Case 7 had normal growth factor concentrations, it should be noted that IGF-I concentrations are sensitive to nutrition[196] and, during infancy, do not reliably identify infants with GHD due to low IGF-I concentrations being normal in infancy[124]. This infant also had low random GH measurements on days 3 (1.11 ng/ml) and 14 (0.334

ng/ml) of life, also supporting the diagnosis of GHD[197]. Case 8 had a diagnosis of focal hyperinsulinism based on a known pathogenic *ABCC8* mutation and previous fasting evaluations consistent with hyperinsulinism. The diagnostic evaluation presented in table 1.4.2 was from an evaluation after the focal lesion had been removed and the hyperinsulinism had resolved. Although his peak GH concentration did not exceed the threshold of 7 ng/ml, his linear growth pattern was not consistent with GHD and a decision was made to observe his growth rather than initiate treatment.

Table 1.4.2: Details of the ten children with peak growth hormone concentrations less than 7 ng/ml after fasting study and glucagon administration.
*Additional clinical data supporting diagnosis of GHD: Height velocity 3 cm/yr, bone age delayed by 2 years (-2.3 SD from mean), improved growth and no further hypoglycaemia following GH treatment.
GH = Growth Hormone, GHD = Growth Hormone Deficiency, HI = Diazoxide Responsive Hyperinsulinism, KH = Ketotic Hypoglycaemia, AI = Adrenal Insufficiency, NA = Data not available, NP = Test not performed

Patient	1	2	3	4	5	6	7	8	9	10
Age, yrs	7.5	1.2	5.3	1.8	1.4	5.1	0.1	2.6	6.1	0.7
Sex	male	male	female	male	female	female	male	male	female	male
Height, z-score	-1.2	-0.81	-2.33	-2.2	-4.1	-0.75	-0.02	-2.47	-3.6	-0.94
Midparental Height, z-score	-0.43	NA	-0.68	0.24	-0.87	-0.46	NA	NA	-0.48	0.25
MRI Brain and Pituitary	normal	NP	NP	normal	NP	NP	abnormal corpus callosum	NP	ectopic neuro hypophysis	normal
Fast Duration, hours	24	15	20	17	10	16	4	13	14	10
Critical Sample										
Glucose (mg/dL)	41	45	49	47	43	51	46	53	45	52
Betahydroxybutyrate (mmol/L)	2.9	1.2	2.2	2.9	2.5	4	0.1	2.5	0.8	2.3
Growth Hormone, ng/ml	3.6	1	1.1	2.4	3.5	3.5	0.8	2.4	0.1	3.2
Cortisol, mcg/dL	20.8	14.4	28.9	9.3	14.3	40.7	14.9	16.3	6	20.6
Peak GH after glucagon (ng/mL)	5.4	5.2	5.5	5.1	4.7	5.7	4.3	4.7	0.3	6.6
Peak GH on repeat GH stimulation test (ng/mL)	7.1	4.6		6.5	3.7	8.2	1.9	6.2	0.4	1.5
Thyroid function tests	normal	NP	normal	normal	normal	NP	normal	normal	normal	normal
IGF-I z-score	-1.4	NP	0.1	-0.9	-3.1	NP	0.7	-1.5	-2.6	-0.7
IGFBP-3 z-score	-1.6	NP	-0.74	-0.18	-1.6	NP	0.77	-0.73	NP	-2
Diagnosis	GHD*	HI	KH	GHD	GHD	KH	GHD	Resolved focal HI	GHD, AI	GHD

A receiver operating characteristics curve showing the effect of adding serial GH measurements to the diagnostic fasting test for children with hypoglycemia

suspected GHD is shown in Figure 1.5.4.. Adding serial GH measurements increased the area under the curve from 0.77 (95% CI 0.57-0.96) to 0.83 (95% CI 0.63-1).

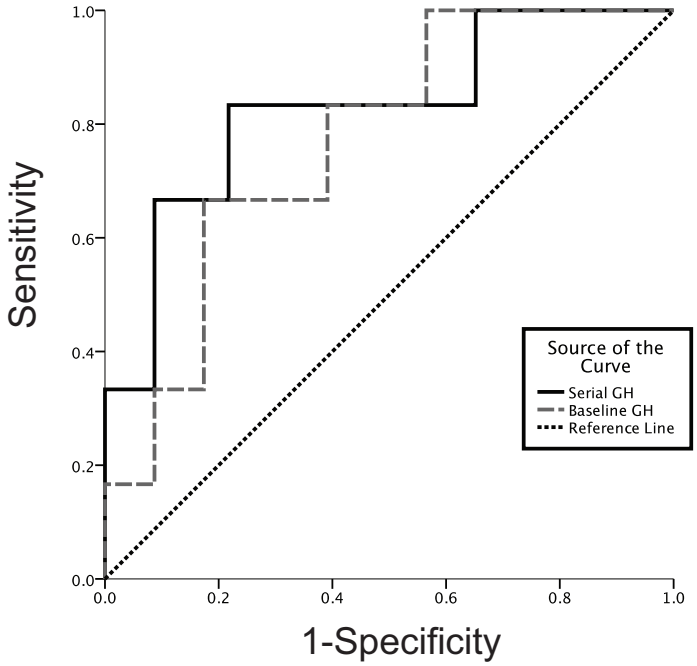

Figure 1.5.4: Receiver-Operating Characteristics curve demonstrating an increased area under the curve when serial GH measurements are used in addition to baseline GH measurement during hypoglycemia.

1.4.2.4 DISCUSSION

We have shown that serial measurement of GH following the administration of glucagon in the context of a fasting study can be a useful adjunct in children suspected of having GHD. GH measurement during hypoglycaemia has poor specificity for GHD and by adding serial GH measurements following glucagon administration, the number of children identified with peak GH concentrations above the arbitrary threshold of 7 ng/ml increased by 16 (55%). This resulted in a more focused evaluation of GH secretion in a smaller number of children than would otherwise have been performed.

The poor specificity of GH measurement during hypoglycaemia has previously been described. In a study including 84 children evaluated for unexplained

hypoglycaemia, only 30% had peak GH concentrations above 7.5 ng/ml[12]. In our study, there was a lower number (10%) of children with peak GH concentration above 7 ng/ml during hypoglycaemia. However, additional GH measurements were only performed in children for whom there was clinical suspiction that GHD was the etiology of their hypoglycaemia, and this selection bias may have contributed to the discrepency in results between our baseline results and previously reported data.

One limitation to adapting our clinical protocol was the blood volume required for additional GH measurements in small infants with unexplained hypoglycaemia. Serial glucose measurement during the diagnostic fast, in addition to the critical sample can limit the blood volume that can be extracted for further testing. Previous studies suggest that the peak GH concentration following glucagon administration (glucagon stimulation test for GH reserve) generally occur after 90 and 120 minutes[190, 192, 198, 199]. Where necessary, GH samples were prioritised at 90 and 120 minutes in this study, and we have shown that these are the most useful measurements in this context to identify GH sufficient children (Figure 1.5.3). Although not evaluated in this study, we note that cortisol responses to glucagon administration occur later, at 150 and 180 minutes[191, 199]. This should be considered if future studies of this test are be adapted to evaluate cortisol response to glucagon in this context.

The mechanism of glucagon-induced GH secretion is not clear. Fluctuations in blood glucose following glucagon administration may contribute to GH secretion, although recent studies suggest that this may not be necessary[190, 200]. Glucagon administration increases noradrenaline secretion[201], which may play a role in stimulating GH secretion. However, alpha adrenergic blockade does not prevent glucagon-induced GH secretion[202]. Although fasting increases GH secretion[203, 204], it is not clear if allowing the patient to feed while measuring GH concentrations after glucagon administration would affect the ability of the test to identify children with GH concentrations above the stimulation threshold. A

large proportion (17/27) of children in this study had an appropriate stimulated GH response to glucagon despite being allowed to feed.

We do not know if the serial GH response noted in this study would have been seen if glucagon was not administered. Hypoglycaemia is a strong stimulus for GH secretion in the absence of additional pharmacological stimuli, and this is utilised in the commonly used insulin tolerance test of GH secretion. However, unlike the induced hypoglycaemia in the insulin tolerance test, Hussain *at al* have shown that the GH response to spontaneous hypoglycaemia in children is blunted[185]. This makes the GH concentrations seen in this study more likely to be secondary to glucagon administration rather than hypoglycaemia alone. We also acknowledge that an intramuscular injection of glucagon may result in higher detectable concentrations of GH relative to intravenous glucagon[205], possibly as a result of an additional painful stimulus increasing GH secretion[206]. As intramuscular or subcutaneous glucagon are more potent stimuli of GH secretion[205, 207, 208], it is possible that modifying the protocol to utilise these routes of administration would further improve the specificity for GHD. However, this route of glucagon administration is not routinely used in evaluating the glycaemic response to hypoglycaemia in our practice. Thus, we are unable to compare different routes of glucagon administration in this study.

It is important to note that many normal children will be characterised as having GHD on GHST alone[47-49], and these results should be interpreted in the clinical context. Depending on the GH stimulus and GH concentration threshold used, the proportion of normal children who do not reach the "sufficient" threshold can be as high as half[47]. In this study, only children suspected as having GHD at the time of diagnostic fasting study underwent this additional serial GH measurement. Given the poor reliability of the GHST, we do not routinely perform GHST in children with peak GH concentrations below 7 ng/ml where the laboratory tests performed during hypoglycaemia indicate that an alternative diagnoses are more likely.

In conclusion, we have shown that additional GH measurements after glucagon administration following a diagnostic fast can improve the identification of children with stimulated GH concentrations above test thresholds. This test can be performed in addition to the diagnostic fasting study, and does not require prolongation of the fast. We also recommend that children with insufficient responses to glucagon in this setting should further have GHD confirmed by standard GHSTs if there is clinical suspicion of GHD.

1.4.3 CHAPTER CONCLUSION

Although short stature and impaired linear growth are the most common presentations of GHD in children, hypoglycaemia may be the only clinical sign in infancy. Correct diagnosis in this population is of critical importance in preventing adverse developmental complications of recurrent hypoglycaemia. Conversely, incorrectly attributing hypoglycaemia to be caused by GHD in this population may put the infant at risk through failure to treat the true underlying diagnosis. In this chapter, I have described a protocol with the potential to steer the physician away from an incorrect diagnostic path through increasing the specificity of the hypoglycaemia evaluation for GHD. This protocol has been adopted by The Hyperinsulinism Center at The Children's Hospital of Philadelphia, the largest referral centre for children with hypoglycaemia in North America.

Additional approaches to evaluating children for GH sufficiency, relevant to neonates who may have hypoglycaemia, will be discussed in subsequent sections of this thesis. Specifically, I will discuss IGF-I measurement in infancy in chapters 2.2 and 2.4, and will explore the utility of body composition measurement in evaluating growth in these children in chapter 4.2.

SECTION 2

INSULIN-LIKE GROWTH FACTOR MEASUREMENT WITH MASS SPECTROMETRY

CHAPTER 2.1: INTRODUCTION

As discussed in Section 1, the GHST alone has poor sensitivity and specificity for diagnosing GHD. Serum IGF-I concentrations are relatively constant in serum and mirror spontaneous GH secretion[80]. Consequently, IGF-I measurement has a role in the diagnostic evaluation of GHD[8, 9, 72] and in the monitoring of treatment efficacy[56, 209, 210] in children receiving GH replacement therapy.

IGF-I circulates bound to binding proteins and this prolongs its serum half-life. Immunoassays are the mainstay of current IGF-I measurement, but these are subject to interference from circulating binding proteins. In Chapter 2.2, I will review IGF-I measurements in infants and demonstrate wide variability in the reported normal concentrations in this age group between assays.

I will then begin to explore mass spectrometry as a possible solution to binding protein interference. My interest in using mass spectrometry to overcome assay interference started with a clinical study where I showed that, in vitamin D intoxication, 25-hydroxyvitamin D interferes with the radioimmunoassay (RIA) for 1,25-dihydroxyvitamin D measurement (Appendix G)[211]. This study will be briefly summarised in Chapter 2.3, before I explore the utility of mass spectrometry in IGF-I measurement in infancy, and report reference data for this in Chapter 2.4.

This section of the thesis focuses on laboratory measurement of IGF-I. Genetic causes of abnormal IGF-I concentrations will be explored in Section 3, and the interaction between nutrition and the GH / IGF-I axis will be reviewed and explored in Section 4.

CHAPTER 2.2: CHALLENGES IN THE MEASUREMENT OF INSULIN-LIKE GROWTH FACTOR-I

Publication

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Infants with isolated GHD have a normal weight and length at birth. In the neonatal period, hypoglycaemia or a small penis may be the only clinical feature of disease[13, 46] (Chapter 1.4). IGF-I measurement is generally part of the first line screen for GHD, but there is wide variation in the reported IGF-I concentrations in normal infants[124]. Similarly, random GH measurement has been suggested as a screen for GHD in infancy[197]. In this chapter, I will systematically review the reported reference data for IGF-I and GH measurements in the first two years of life with an emphasis on assay variability.

2.2.1 THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS IN INFANCY

GH secretory patterns differ between neonates and older children[212]. Higher GH peaks are seen in the term neonate than the older child. These peaks become less pronounced within the first four days of life, and the frequency of secretory pulses also halves over the same time period[213]. Even higher GH levels are seen in preterm infants, but the pulsatile pattern of release is similar to that of the term infant[214]. Sleep is not a stimulus for GH secretion until three months of age[215, 216], but feeding and insulin release stimulate GH secretion at this early stage before sleep entrainment[217].

The role of GH in the fetus is poorly understood. Although GH is detected in the fetal pituitary gland as early as ten weeks' gestation, and the GH content of the

pituitary gland increases with gestational age[218], GH is not required for normal intrauterine growth[219].

Unlike GH, IGF-I plays a major role in fetal growth. IGF-I levels increase 2-3 fold from 33 weeks' gestation to term,[220] and cord blood levels of IGF-I positively correlate with weight, length and head circumference at birth[221] (Chapter 2.4). Postnatal IGF-I production is involved in both somatic and brain growth, independent of gestational age and caloric intake[222]. Postnatally, IGF-I concentrations and bioavailability correlate with increased growth in low birthweight[223] and preterm infants[224].

Diagnosing GHD in infancy remains a challenge. A combination of clinical phenotype, stimulation testing, imaging and baseline IGF-I, IGFBP-3 and GH levels can be used[72, 212, 225, 226]. Normative data for GH and IGF-I concentrations are limited in the non-GH deficient child under 18 months of age. This can be a challenge when interpreting IGF-I measurements in this age group.

2.2.2 Assays used in Measuring Growth Hormone and Insulin-like Growth Factor-I

2.2.2.1 Growth Hormone Assays

Two isoforms of GH are present in serum, as a consequence of alternative splicing during transcription. These two isoforms differ by the presence of 15 amino acids in the larger 22-kDa isoform. The absence of these amino acids in the 20-kDa isoform makes it more likely to dimerise. This isoform represents 5-10% of circulating GH and is less biologically active than the 22-kDa isoform[227]. Human serum contains hetero- and homodimers, as well as multimers, of these isoforms[228]. This heterogeneity in circulating GH complicates its measurement, as assays may recognise different isoforms and protein structures differently[229, 230].

Bioassays and radioreceptor assays have been developed to determine the biological activity of GH in a serum sample, but they are insensitive and time consuming and thus, not widely used in clinical practice[227]. Commercially available immunoassays are generally used to measure serum concentrations of GH, and the particular assay used may differ between centres[231, 232].

The two main types of immunoassays are the competitive immunoassay and the sandwich type immunoassay. In competitive assays, labeled GH is added to serum and competes with the sample's unlabeled GH for binding sites on an antibody-coated platform. The concentration of bound labeled GH can be used to determine the concentration of unlabeled GH in the sample. Sandwich type immunoassays use two different antibodies that bind to different epitopes of the GH molecule and are detected using radioactive, enzyme linked or chemiluminescent labels[233] (Chapter 2.3.1). The method of detection determines the specific assay type: RIA; Enzyme-linked ImmunoSorbent Assay (ELISA) or Chemiluminescent assays (ECL).

Another difference between assays is the type of antibodies used. The antibodies can be directed at one epitope (monoclonal) or multiple epitopes (polyclonal) on the GH molecule[233]. This will restrict the comparability of results between assays, as polyclonal assays tend to recognise more isoforms and yield higher results[233].

2.2.2.2 IGF-I Assays

Free IGF-I represents a small percentage of total IGF-I in the serum, with the majority forming a 150-kDa ternary complex with IGFBP-3 and acid labile subunit (ALS). Some IGF-I also circulates in binary complexes with the various IGFBPs[234]. In order to utilise immunoassays to measure IGF-I, IGF-I must first be separated from the complexes, and this process can result in significant inter- and intra-assay variability. The most commonly used technique for separating large molecular weight molecules involves ethanol extraction. While this dissociates IGF-I from its binding proteins and precipitates large molecules from

the sample, IGFBP-1 and IGFBP-4 do not precipitate well and may affect results[235]. Other techniques such as IGF-II saturation can be used to saturate the binding sites on the IGFBPs and thereby reduce the unmeasured IGF-I[235-237]. This can be combined with ethanol precipitation to further reduce the interference of binding proteins in IGF-I measurement.

Like the GH assays, antibodies used for IGF-I measurement can be polyclonal or monoclonal. Where IGF-II saturation has been used, cross reactivity of the antibody with IGF-II must be very low or the results will be falsely elevated.

Liquid chromatography mass spectrometry (LCMS) is a new method of measuring IGF-I (and –II) that is not affected by binding protein interference[238] (See 2.4.3.5 for details). This method is becoming increasingly available and may represent the future of IGF-I measurement[238-241].

2.2.3 Measurements of Growth Hormone and Insulin-like Growth Factor-I in Children aged 0-18 months

2.2.3.1 Literature Review Methodology

A PubMed search was performed for "growth hormone" OR "IGF-I" in November 2012, limited from birth to 18 months. Titles were used to identify articles that were likely to include GH or IGF-I measurement in the fetus or infant less than 18 months of age. Abstracts were then read to determine if these articles included GH or IGF-I measurement of healthy children, and full articles were then accessed to determine a) assay used and b) reported values of GH and/or IGF-I concentrations.

Initial PubMed search identified 4451 articles. 525 articles were identified as relevant from screening titles. GH was measured in 44 of these articles in healthy children less than 18 months of age, while IGF-I was measured in 113. Relevant articles that reported mean values with standard deviations were included in this

review. Unless otherwise stated, values in this manuscript are presented as mean ± 1 standard deviation.

2.2.3.2 Growth Hormone

Due to the pulsatile nature of GH secretion, timing is important in the interpretation of GH measurements. Except for the first 15 days of life, random GH measurement is of limited utility. Most studies in which this has been reported have shown a mean GH concentration below most accepted cutoff values for GH stimulation tests. This appears to be consistent regardless of assay used (Table 2.2.1).

Random GH measurement within the first 15 days of life may, however, be clinically useful. There is a GH surge at birth, and the lowest reported mean umbilical cord GH measurement in all assays was 9.2 ng/ml[242, 243]. Cord GH concentration is higher in pregnancies complicated by preeclampsia[244]. Random GH rises further on day two[245, 246] and remains elevated up to day five, with mean values of 53.4 ± 30.8 ng/ml (IRMA)[247] or 21.8 ± 11.2 ng/ml (IFMA)[224]. By day 15, the mean falls to 5.5 ± 3.7 ng/ml (IFMA) [224] and by 3 weeks a mean of 8.7 ± 8.1 ng/ml (RIA)[248] was shown. Assay variability makes comparison of values difficult, but a temporal trend is observed in all studies. By one[224] and two[248] months of age, low mean random GH values consistent with those seen in older children are seen.

Serial measurements of GH have described the secretory pattern of GH in infancy. Pulsatile secretion is also present during this early period of raised GH levels. When GH was measured every 20 minutes for 6 hours on the first day of life (by RIA with a polyclonal antibody), values ranged between 9 and 191 ng/ml. Five to six bursts of GH release occurred within the six hour study period[249]. Similarly, GH measurement every thirty minutes for 12 hours in infants ranging in age from 8 to 40 hours varied from 4.1 to 105 ng/ml using a double antibody RIA[213]. In slightly older infants, aged between one and four

days, GH burst frequency was 7.7 over a 6 hour period[214]. This is similar to the frequency seen in infants on day one of life.

With such a wide range of GH concentrations found on serial measurement in infancy, it would be clinically useful to identify a time when GH secretion is likely to peak. This would allow for optimally timed single sample measurement to identify GH sufficiency in infants where there is a clinical concern regarding possible GH deficiency. At two days of age, mean circulating GH concentrations measured by RIA after five minutes of active sleep, quiet sleep and waking were 33.4 ± 7, 52.8 ± 11 and 43.5 ± 36 ng/ml, respectively. These high mean values were not seen between four and eight days of age, with respective values of 9.1 ± 1.1, 9.5 ± 1.3 and 8.3 ± 1.2 ng/L noted[250], and may represent the higher GH levels in the first few days of life. Sleep entrainment of GH secretion does not occur until after three months of age.[215] When GH concentrations were measured by RIA following 30 minutes of sleep and 30 minutes of wakefulness at different ages within the first six months of life, mean sleep and waking values were similar until three months of age. Mean awake/asleep GH concentrations were 20.6 ± 10.9 / 17.6 ± 9.1 ng/ml at 1-4 weeks, 17.1 ± 11.6 / 17.4 ± 10.7 ng/ml at 1-3 months, 6.5 ± 3.4 / 15.1 ± 13.1 ng/ml at 3-6 months and 6.2 ± 3 / 16.6 ± 9.7 ng/ml at 6-12 months[215].

Table 2.2.1: Random GH Measurement within the first 18 months of life

Author	Timing	Population	Number	Assay	-2SD	Mean (ng/ml)	+2SD
ELISA							
Leger 1996[251]	Umbilical Cord	Term infants	44	Solid phase two-site Immunoradiometric assay (Elisa-hGH, CIS bio International)	1	19	37
Setia 2007[252]	Umbilical Cord	Term infants	50	Solid phase immunoradiometric assay	1	16.2	31.4
Binder 2010[225]	Day 2-4	Term infants	269	hGH-ELISA (Mediagnost, Reutlingen, Germany). Polyclonal Ab against 22kDa rhGH in rabbit. Measured from dried bloodspots on filter paper		16.6	
Radioimmunoassay							
Desgranges 1987[253]	Umbilical Cord	Term infants	18	Radioimmunoassay Double-antibody incubation	4.2	17.4	30.6
Geary 2003[254]	Umbilical Cord	Term infants	1197	Radioimmunoassay – (Hybritech Europe, Liege, Belgium)	0	10.5	27.5
Kitamura 2003[255]	Umbilical Cord	Term infants	54	Radioimmunoassay (Daiichi Rasioisotope Laboratories, Tokyo, Japan)		17.8	
Cornblath 1965[246]	Umbilical Cord	Term infants	20	Radioimmunoassay	0	66	210.4
Laron 1967[244]	Umbilical cord	Term infants	9	Radioimmunossay		34	
Cornblath 1965[246]	Day 1	Term infants	15	Radioimmunoassay	0	52	130.6
Laron 1966[245]	Day 1	Term males	36	Radioimmunoassay		38.4	
Laron 1967[244]	Day 1	Term infants	9	Radioimmunoassay		69	
Laron 1966[245]	Day 1	Term females	25	Radioimmunoassay		50	
Cornblath 1965[246]	Day 2	Term infants	13	Radioimmunoassay	0	72	184.6
Laron 1966[245]	Day 2-3	Term males	36	Radioimmunoassay		76.6	
Laron 1966[245]	Day 2-3	Term females	25	Radioimmunoassay		54	
Cornblath 1965[246]	Day 2-6	Term infants	20	Radioimmunoassay	0	32	70.6
Laron 1966[245]	Day 4-5	Term males	36	Radioimmunoassay		19.3	
Laron 1966[245]	Day 4-5	Term females	25	Radioimmunoassay		26.2	
Cassio 1998[248]	3 weeks	Term infants	12	Commercial liso-solid phase radioimmunoassay (Technogenetics, Milan, Italy)	0	8.7	24.9
Cornblath 1965[246]	3-4 weeks	Term infants	8	Radioimmunoassay	0.8	16	31.2
Cassio 1998[248]	4 months	Term infants	8	Commercial liso-solid phase radioimmunoassay (Technogenetics, Milan, Italy)	0	2.8	5.8
Cassio 1998[248]	7 months	Term infants	10	Commercial liso-solid phase radioimmunoassay (Technogenetics, Milan, Italy)	0.2	2	3.8

Table 2.2.1 (continued)

Author	Timing	Population	Number	Assay	-2SD	Mean (ng/ml)	+2SD
IRMA							
Chiesa 2008[256]	Umbilical Cord	AGA Term neonates	87	IRMA (CIS Bio International, Schering S.A.)	14.6	18.2	21.8
Cance-Rouzaud 1998[247]	Within first 5 days of life	AGA Term infants	64	Immunoradiometric assay Protein binding to a first antibody was revealed by a second ^{125}I-labelled antibody (ELISA kit, CIS-Bio International, Gif-sur-Yvette, France)	0	53.4	115
Leger 1996[251]	6 months		12	Solid phase two-site Immunoradiometric assay (Elisa-hGH, CIS bio International)	0	3.4	8.2
Leger 1996[251]	12 months		37	Solid phase two-site Immunoradiometric assay (Elisa-hGH, CIS bio International)	0	2.7	7.1
Immunofluorometric Assay							
Pagani 2007[224]	Day 5, (8-9am)	AGA Term infants	26	Immunoflurometric assay. Direct sandwich technique. AutoDELFIA hGH Kit (Wallacoy, Turku, Finland)	0	21.8	44.2
Pagani 2007[224]	Day 15, (8-9am)	AGA Term infants	26	Immunoflurometric assay. Direct sandwich technique. AutoDELFIA hGH Kit (Wallacoy, Turku, Finland)	0	5.5	12.9
Pagani 2007[224]	Day 30, (8-9am)	AGA Term infants	26	Immunoflurometric assay. Direct sandwich technique. AutoDELFIA hGH Kit (Wallacoy, Turku, Finland)	0	1.8	4.2
Chemiluminescent immunometric assays							
Chanoine 2003[242] and 2002[243]	Umbilical Cord	AGA Term neonates	90	Chemiluminescent immunoassay (Beckman Coulter, Fullerton, Calif, USA)	0	9.2	35
Gesteiro 2009[257]	Umbilical Cord	Term infants	115	Cheminuminescent immunometric assays (Diagnostic Products Corporation, Flanders, New Jersey) Ref No LKGH1	0	16.7	36.3
Osmanagaoglu 2005[258]	Umbilical Cord	AGA	50	Two-site chemiluminescent enzyme immunometric assay (Immulite 1000 human GH kit, DPC, Los Angeles, CA, USA)	7	11.6	16.2
Osmanagaoglu 2005[258]	Umbilical Cord	SGA	60	Two-site chemiluminescent enzyme immunometric assay (Immulite 1000 human GH kit, DPC, Los Angeles, CA, USA)	1.8	11.6	21.4
Osmanagaoglu 2005[258]	Umbilical Cord	LGA	50	Two-site chemiluminescent enzyme immunometric assay (Immulite 1000 human GH kit, DPC, Los Angeles, CA, USA)	3.2	12.2	21.2

2.2.3.3 Insulin-Like Growth Factor-I

Serum IGF-I levels reflect spontaneous 24-hour GH secretion[80] and, unlike GH, random levels are relatively stable[259]. IGF-I measurements are sensitive to extraction methods and assays used. Many studies identified in this review did not provide details regarding the extraction methods used, and this may account for the variation in reported values in studies where the same assay was used to measure IGF-I levels in children of similar ages and stature[260]. Tables 2.2.2, 2.2.3 and 2.2.4 summarise the IGF-I values reported in studies including healthy children aged less than 18 months.

Factors that may affect IGF-I concentrations in cord blood include ethnicity, maternal smoking, and some antenatal medication exposure. Chinese infants have higher IGF-I concentrations than Caucasian infants[261] while Caucasian infants may have a higher IGF-I concentration than African American infants[262]. Maternal smoking may be associated with lower IGF-I levels in cord blood, even where infant sizes are similar[263]. Selective serotonin reuptake inhibitor use in pregnancy may be associated with lower umbilical cord IGF-I levels[264]. Many of these studies, however, involved small sample sizes and statistical significance was not demonstrated.

In studies where infants and children were categorised according to weight, the group of larger children consistently had higher IGF-I concentrations regardless of assay used. Some studies have shown that girls have higher IGF-I concentrations than age-matched boys with a similar weight, at birth, as well as at 12 and 24 months of age[254, 265-268]. While the etiology of this difference in IGF-I concentrations between sexes is not clear, it may be related to differences in body composition between males and females that are not captured in weight measurement. At birth, girls have been shown to have a greater proportion of body fat than boys, even when boys are heavier[269, 270].

Table 2.2.2: Assay Specific IGF-I measurement in umbilical cord blood

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
ELISA									
Akram 2011[271]	Umbilical cord	SGA	12	<2.5	ELISA (Mediagnost, Germany).		5.77	6.35	6.93
Akram 2011[271]	Umbilical cord	AGA	21	>3.5	ELISA (Mediagnost, Germany).		8.86	14.08	19.6
Jayanthiny 2011[272]	Umbilical cord	Term Infants	200		Enzyme linked immunosorbent assay (Catalogue No. EIA-2947, DRG International, Inc., USA)		5.9	39	72
Rohrmann 2009[262]	Umbilical cord	African American Infants	75	3.2 (2.5-3.9)	ELISA (Diagnostic Systems Laboratory)		0	72.3	156.3
Rohrmann 2009[262]	Umbilical cord	Caucasian Infants	38	3.5 (2.7-4.3)	ELISA (Diagnostic Systems Laboratory)		0	90.6	208.2
Radioimmunoassay									
Bankowski 2000[273]	Umbilical cord	Term Infants	12	3.53 (2.8-4.2)	Radioimmunoassay	Acid Chromatography		130	
Beltrand 2008[274]	Umbilical cord	Highest antenatal growth tertile	78	3.18 (2–4.4)	Radioimmunoassay		0	85.6	182.8
Beltrand 2008[274]	Umbilical cord	Middle antenatal growth tertile	78	2.97 (1.7–4.3)	Radioimmunoassay		0	65.7	137.1
Beltrand 2008[274]	Umbilical cord	Lowest antenatal growth tertile	79	2.8 (2–3.6)	Radioimmunoassay		0	61.6	180.6
Bennett 1983[275]	Umbilical cord	Term Infants	32		Radioimmunoassay		41	113	185
Halhali 2000[276]	Umbilical cord		20	3.2 (2.5-3.8)	Radioimmunossay . (Nichols Institute Diagnostics)	Acid ethanol extraction	19	123	227
Klauwer 1997[277]	Umbilical cord	Term Infants	138	3.3 (2.2-4.4)	Radioimmunoassay	Acid extraction Excess IGF-II	19	61	103
Leger 1996[251]	Umbilical cord	Term Infants	44	2.9 (1.5-4.3)	Radioimmunoassay using polyclonal IGF-I antiserum	Acid chromatography	13	85	157
Simmons 1995[278]	Umbilical cord		120	3.5 (2.3-4.5)	Radioimmunoassay (Ciba-Geigy, Basel, Switzerland)	Ethanol	8.7	57.1	105.5
Vatten 2002[279]	Umbilical cord		609		Radioimmunoassay (Mediagnost, Tuebingen, Germany)		10	64	118
Wang 2005[266]	Umbilical cord	Males	62	3.2 (2.4-4)	Radioimmunoassay (Daiichi Radioisotope Laboratory, Tokyo, Japan)		14.9	84.3	153.7
Wang 2005[266]	Umbilical cord	Females	57	3.1 (2.3-3.9)	Radioimmunoassay (Daiichi Radioisotope Laboratory, Tokyo, Japan)		20	95.2	170.8
Wiznitzer[280]	Umbilical cord	AGA	20	3.3 (2.3-4.3)	Radioimmunoassay		16	80	144
Wiznitzer[280]	Umbilical cord	LGA	40	4.3 (3.6-4.8)	Radioimmunoassay		5	139	273
IRMA									
Baik 2006[281]	Umbilical cord	Term Infants	30		IRMA (Diagnostic Systems Lab)		0	54.3	128.5
Chiesa 2008[256]	Umbilical cord	Term Infants	87	3.19 (2.6–3.8)	IRMA (Immunotech)		0	99	203.7
Christou 2001[282]	Umbilical cord	AGA	96	3.2 (3.1–3.3)	IRMA (Diagnostic Systems Lab)		0	43.4	109.6
Christou 2001[282]	Umbilical cord	LGA	46	4 (3.9-4.1)	IRMA (Diagnostic Systems Lab)		0	73.8	153.4

Table 2.2.2 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Cooley 2004[283]	Umbilical cord	Term Infants	1650	3.4 (2.3-4.5)	IRMA (Nichols Institute Diagnostics)	Acidification and IGF-II excess	22.8	217.5	412.2
Davidson 2009[264]	Umbilical cord	Antenatal SSRI exposure	21	3.2 (3–3.4)	IRMA (Diagnostic Systems Lab)		66.9	96.1	125.3
Davidson 2009[264]	Umbilical cord	Term Infants	20	3.3 (3.2–3.5)	IRMA (Diagnostic Systems Lab)		93.1	119.9	146.7
Geary 2003[254]	Umbilical cord	Males	515	3.5 (3.4-3.6)	IRMA (Nichols Institute Diagnostics)	Acidification and IGF-II excess	64	66.4	68.8
Geary 2003[254]	Umbilical cord	Females	472	3.5 (3.4-3.6)	IRMA (Nichols Institute Diagnostics)	Acidification and IGF-II excess	71.9	74.5	77.1
Ingvarsson 2007[263]	Umbilical cord	Maternal cigarettes	30	3.9 (2.9-4.9)	Two site IRMA (Active™ IGF-I)	Yes	0	54.4	119.4
Ingvarsson 2007[263]	Umbilical cord	No maternal cigarettes	60	3.4 (2.4-4.5)	Two site IRMA (Active™ IGF-I)	Yes	0	93.8	202.8
Lagiou 2009[284]	Umbilical cord	Caucasian American Infants	92	3.6 (2.6-4.5)	Immunoradiometric assay kit (Diagnostic Systems Laboratories, Webster, Texas)		22.8	98.4	174
Lagiou 2009[284]	Umbilical cord	Chinese Infants	110	3.5 (2.6-4.5)	Immunoradiometric assay kit (Diagnostic Systems Laboratories, Webster, Texas)		0	79	177
Lo 2002[285]	Umbilical cord		43	3.2 (2.4-4)	IRMA (Diagnostic Systems Laboratories, Webster, Texas)		13.1	66.5	119.9
Maffeis 1999[286]	Umbilical cord	Males	48		Immunoradiometric assay (CIS Bio International, Gif-sur-Yvette, France)		30	87.4	144.2
Maffeis 1999[286]	Umbilical cord	Females	50		Immunoradiometric assay (CIS Bio International, Gif-sur-Yvette, France)		0	73.9	148.9
Martinez-Cordero 2006[287]	Umbilical cord	SGA	50	2.3 (2-2.6)	Immunoradiometric assay (Biocode-Hycel, Liege, Belgium)		15.4	71	126.6
Martinez-Cordero 2006[287]	Umbilical cord	AGA	50	3.8 (3-4.5)	Immunoradiometric assay (Biocode-Hycel, Liege, Belgium)		0	76.5	154.7
Pringle 2005[288]	Umbilical cord		1192	3.5 (2.5-4.4)	Immunoradiometric assay (Nichols Institute Diagnostics, San Juan, Capistrano CA)	Acidification Excess IGF-II	17.7	68.5	119.3
Chemiluminescent immunometric assays									
Gesteiro 2011[289]	Umbilical cord	Lowest quartile for cord insulin	33	3.2 (2.5-3.9)	Chemiluminescent immunometric assays (Diagnostic Products Corporation)		7	46.8	86.7
Gesteiro 2011[289]	Umbilical cord	Second quartile for cord insulin	43	3.3 (2.6-3.9)	Chemiluminescent immunometric assays (Diagnostic Products Corporation)		10	52.6	103.2
Gesteiro 2011[289]	Umbilical cord	Third quartile for cord insulin	44	3.4 (2.7-4)	Chemiluminescent immunometric assays (Diagnostic Products Corporation)		17.5	64.2	111
Gesteiro 2011[289]	Umbilical cord	Highest quartile for cord insulin	56	3.4 (2.7-4)	Chemiluminescent immunometric assays (Diagnostic Products Corporation)		0	63.7	128.3

Table 2.2.2 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Gesteiro 2009[257]	Umbilical cord		109	3.3 (2.7-4)	Chemiluminescent immunometric assays (Diagnostic Products Corporation)		9.4	55.2	101
Ibanez 2008[267]	Umbilical cord	SGA females	24	2.3 (1.9-2.7)	Immunochemiluminescent assay (Immulite 2000; Diagnostic Products, Los Angeles, CA)		10	59	108
Ibanez 2008[267]	Umbilical cord	AGA males	24	3.4 (3-3.8)	Immunochemiluminescent assay (Immulite 2000; Diagnostic Products, Los Angeles, CA)		5.2	64	122.8
Ibanez 2008[267]	Umbilical cord	AGA females	24	3.4 (3-3.8)	Immunochemiluminescent assay (Immulite 2000; Diagnostic Products, Los Angeles, CA)		35	84	133
Ibanez 2008[267]	Umbilical cord	SGA males	24	2.3 (1.9-2.7)	Immunochemiluminescent assay (Immulite 2000; Diagnostic Products, Los Angeles, CA)		1.8	41	80.2
Kyriakakou 2009[290]	Umbilical cord	IUGR	20	2.4 (1.9-2.9)	2 site chemiluminescence immunoassay (Nichols Diagnostic Institute, San Juan Capistrano, CA)		17.6	42.6	67.6
Kyriakakou 2009[290]	Umbilical cord	AGA	20	3.2 (2.6-3.7)	2 site chemiluminescence immunoassay (Nichols Diagnostic Institute, San Juan Capistrano, CA)		19.6	45.5	71.4
Luo 2012[291]	Umbilical cord		229	3.4 (2.5-4.3)	Enzyme-labeled chemiluminescent assay	Acid buffer dilution	8.5	60.5	112.5
Troisi 2008[261]	Umbilical cord	Caucasian Infants	51	3.6	Direct chemiluminescent immunoassay. Immulite analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, CA)		0	76.3	421.7
Troisi 2008[261]	Umbilical cord	Chinese Infants	22	3.5	Direct chemiluminescent immunoassay. Immulite analyzer (Siemens Medical Solutions Diagnostics, Los Angeles, CA)		0	90.1	360.5

Table 2.2.3: Assay Specific IGF-I measurement 0-6 months of age. Blank cells represent unreported data.

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
ELISA									
Yuksel 2011[265]	3 months	Males	29		ELISA (Diagnostic Systems Laboratories Inc)		0	110.8	279.8
Yuksel 2011[265]	3 months	Females	22		ELISA (Diagnostic Systems Laboratories Inc)		0	111.3	290.7
Yuksel 2011[265]	6 months	Males	41		ELISA (Diagnostic Systems Laboratories Inc)		0	89.3	286.7
Yuksel 2011[265]	6 months	Females	33		ELISA (Diagnostic Systems Laboratories Inc)		0	67.8	216
Radioimmunoassay									
Gunes 2007[292]	Day 1	Mothers who smoke	28	3.02 (2.7-3.3)	Radioimmunoassay Biocode 1010 (Liege Belgium)		107.6	156.6	205.6
Gunes 2007[292]	Day 1	Mothers who don't smoke	28	3.5 (2.94-4)	Radioimmunoassay Biocode 1010 (Liege Belgium)		104	164.4	224.8
Koklu 2007[293]	Day 1	LGA, mothers with diabetes	30	4.3 (3.9-4.7)	Radioimmunoassay (Biocode 1010, Liege, Belgium)		85	205.2	325.4
Koklu 2007[293]	Day 1	LGA	30	4.3 (3.9-4.7)	Radioimmunoassay (Biocode 1010, Liege, Belgium)		69.1	179.3	289.5
Koklu 2007[293]	Day 1	AGA	30	3.6 (3.2-4)	Radioimmunoassay (Biocode 1010, Liege, Belgium)		47	113.2	179.4
Koklu 2007[294]	Day 1	IUGR	40	2.4g (2.1-2.8)	Radioimmunoassay (Biocode 1010, Liege, Belgium)		33.6	75.4	117.2
Koklu 2007[294]	Day 1	AGA	40	3.6 (3.2-4)	Radioimmunoassay (Biocode 1010, Liege, Belgium)		24.4	90.8	157.2
Rajaram 1995[295]	Day 1		51	3.3 (2.5-4.1)	Radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA)		49.7	59.1	68.5
Iniguez 2006[296]	Day 2	SGA			Locally developed radioimmunoassay	With extraction	23.5	41.9	60.3
Iniguez 2006[296]	Day 2	AGA			Locally developed radioimmunoassay	With extraction	41.6	59.6	77.6
Leger 1996[251]	Day 3		36		RIA using polyclonal IGF-I antiserum	Acid chromatography	0	66	138
Cassio 1986[297]	Day 3	SGA, Term	11	2.6 (1.9-3.3)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	18	39
Cassio 1986[297]	Day 3	AGA, Term	198	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	18	39

Table 2.2.3 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Cassio 1986[297]	Day 4	SGA, Term	48	2.6 (1.9-3.3)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	7.5	19.5	31.5
Cassio 1986[297]	Day 4	AGA, Term	417	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	4	19.5	34.5
Cassio 1986[297]	Day 5	SGA, Term	23	2.6 (1.9-3.3)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	19.5	43.5
Cassio 1986[297]	Day 5	AGA, Term	222	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	24	48
Cassio 1986[297]	Day 6	AGA, Term	49	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	28.5	58.5

Table 2.2.3 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Cassio 1986[297]	Day 7	AGA, Term	32	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	4.5	28.5	52.5
Mitchell 1989[298]	1 week		99		Filter paper eluted in phosphate buffer containing rabbit anti-human IGF-I antibody, heparin and radiolabelled IGF-I overnight.	Goat antirabbit gamma-globulin in 6% polyethylene glycol.	0	13.5	31.5
Cassio 1986[297]	Day 7-15	AGA, Term	50	3.4 (2.2-4.6)	Radioimmunoassay (Nichols Institute Company reagents) on capillary samples via heel prick	Heel prick samples on filter paper. Eluted in borosilicate tubes with protamine-free buffer. Incubated 1hr with antiserum, and 16-20hr with ^{125}I-labelled SmC.	0	45	105
Mitchell 1989[298]	2-3 weeks		42		Filter paper eluted in phosphate buffer containing rabbit anti-human IGF-I antibody, heparin and radiolabelled IGF-I overnight.	Goat antirabbit gamma-globulin in 6% polyethylene glycol.	3	54	105
Cassio 1998[248]	3 weeks		12		Radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA)		0	68.2	175
Leger 1996[251]	1 month		51		RIA using polyclonal IGF-I antiserum	Acid chromatography	20	90	160
Cassio 1998[248]	2 months		6		Radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA)		57.6	116	174.4
Rajaram 1995[295]	2 months		51	Birthweight 3.3 (2.5-4.1)	Radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA)		49	55.2	61.4
Chellakooty 2006[299]	3 months	AGA, Term	942	Birthweight 3.6	Radioimmunoassay	Extracted by acid/ethanol and cryoprecipitated before analysis to remove binding proteins. Monoiodinated Tyr31 IGF-I used as radioligand.		92	

Table 2.2.3 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Chellakooty 2006[299]	3 months	SGA, Term	49	Birthweight 2.5	Radioimmunoassay	Extracted by acid/ethanol and cryoprecipitated before analysis to remove binding proteins. Monoiodinated Tyr31 IGF-I used as radioligand.		88	
Kai 2006[300]	3 months	Male		Birthweight 3.6 (2.6-4.6)	Radioimmunoassay . Monoiodinated Tyr IGF-I used as radioligand)	Acid/ethanol extraction and cryoprecipitation	39.6	94	148.4
Kai 2006[300]	3 months	Female		Birthweight 3.5 (2.5-4.5)	Radioimmunoassay . Monoiodinated Tyr IGF-I used as radioligand)	Acid/ethanol extraction and cryoprecipitation	40.2	93	145.8
Leger 1996[251]	3 months		16		RIA using polyclonal IGF-I antiserum	Acid chromatography	20	78	136
Ong 2009[301]	3 months old	Breastfed	61 boys 57 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	11.3 8.1	Boys 43.7 Girls 37.3	76.1 66.5
Ong 2009[301]	3 months old	Formula fed	92 boys 68 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	12.2 8.8	Boys 55.4 Girls 51	98.6 93.2
Cassio 1998[248]	4 months		8		Radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA)		4	77.2	150.4
Rajaram 1995[295]	4 months		51	Birthweight 3.3 (2.5-4.1)	Radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA)		43.6	49.8	56
Hasegawa 1997[302]	1-6 months		52		Monoclonal radioimmunoassay developed by authors	Acid ethanol extraction	0	90.3	229.5
Leger 1996[251]	6 months		12		RIA using polyclonal IGF-I antiserum	Acid chromatography	30	102	174
Rajaram 1995[295]	6 months		51	Birthweight 3.3 (2.5-4.1)	Radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA)		35	41.2	47.4
Ong 2009[301]	12 months	Breastfed	61 boys 57 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	8 3.6	Boys 38.8 Girls 56	69.6 108.4
Ong 2009[301]	12 months	Formula fed	92 boys 68 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	4.8 7.3	Boys 47.6 Girls 59.9	90.4 112.5
IRMA									
Baker Melo 2009[303]	Day 1	SGA	28	1.5 (0.3-2.7)	IRMA Active IGF-I DSL-5600	IGF-I extraction via HCl ethanol solution. I-125 marked antibody.	0	2.78	7.52
Baker Melo 2009[303]	Day 1	AGA	26	2.4 (1-3.9)	IRMA Active IGF-I DSL-5600	IGF-I extraction via HCl ethanol solution. I -125 marked antibody.	0	3.99	10.07

Table 2.2.3 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Low 2001[304]	Day 1	Term, AGA	48		Immunoradiometric assay (Diagnostic Systems Laboratories Inc, Webster, TX, USA). 2 site assay.		0.8	48.1	95.4
Satar 2004[305]	Day 1		19		Immunoradiometric assay (Immunotech)		20.7	121.5	222.3
Thieriot-Prevost 1988[306]	Day 3		62		Radioimmunoassay	Extraction on a Sep-Pak column using kits from Immuno Nuclear Corporation (Stillwater, MN)	0	48	118.9
Pagani 2007[224]	Day 4	Term, AGA	26		Immunoradiometric assay Diagnostic Systems	Acid ethanol extraction	32.5	51.2	69.9
Cance-Rouzaud 1998[247]	Day 0-5	AGA	64	2.7 (1.3-4)	IRMA (IGF-IRMA DSL-9300 kit, Chiron Diagnostics, Cergy-Pontoise, France)	Ethanol extraction	0	11.7	28.1
Cance-Rouzaud 1998[247]	Day 0-5	SGA	26	1.5 (0.8-2.2)	IRMA (IGF-IRMA DSL-9300 kit, Chiron Diagnostics, Cergy-Pontoise, France)	Ethanol extraction	0	6.6	14.6
Bozzola 1998[307]	Day 5	Term infants	19		Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas)	Acid-ethanol extraction	20.3	27	33.7
Bozzola 1996[308]	Day 5	Term infants	60		Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas)	Acid-ethanol extraction	0	67.6	148.2
Ermis 2004[309]	Day 7 Mothers who smoke		21	3.2 (2.5-3.8)	2 site IRMA (Diagnostic Systems Laboratories, Webster, Texas, USA)	"Sample processed according to manufacturer's instructions"	63	119	175
Ermis 2004[309]	Day 7 non-smoker mothers		23	3.2 (2.4-4)	2 site IRMA (Diagnostic Systems Laboratories, Webster, Texas, USA)	"Sample processed according to manufacturer's instructions"	56	126	196
Satar 2004[305]	Day 10		19		Immunoradiometric assay (Immunotech)		64.3	133.1	201.9
Bozzola 1996[308]	1 month	Term infants	60		Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas)	Acid-ethanol extraction	0	72.6	190.3
Pagani 2007[224]	1 month	Term, AGA	26		Immunoradiometric assay Diagnostic Systems	Acid ethanol extraction	19.1	73.3	127.5
Thieriot-Prevost 1988[306]	1 month		62		Radioimmunoassay	Extraction on a Sep-Pak column using kits from Immuno Nuclear Corporation (Stillwater, MN)	0	75	216.7
Bozzola 1998[307]	3 months	Term infants	19		Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas)	Acid-ethanol extraction	7.4	44.9	82.4
Bozzola 1996[308]	4 months	Term infants	60		Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas)	Acid-ethanol extraction	0	97.9	248.2
Low 2001[304]	6 months	Term, AGA	48		Immunoradiometric assay (Diagnostic Systems Laboratories Inc, Webster, TX, USA). 2 site assay.		0	42	92.4

Table 2.2.3 (continued)

Author	Timing	Population	Number	Weight (kg)	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Chemiluminescent immunometric assays									
Kyriakakou 2009[290]	Day 1 and Day 4	IUGR	20	2.4 (1.9-2.9)	2 site chemiluminescence immunoassay (Nichols Diagnostic Institute, San Juan Capistrano, Calif)		14.4 13	Day 1 22.5 Day 4 22	30.5 30.9
Kyriakakou 2009[290]	Day 1 and Day 4	AGA	20	3.2 (2.6-3.7)	2 site chemiluminescence immunoassay (Nichols Diagnostic Institute, San Juan Capistrano, Calif)		12.8 11.8	Day 1 21.7 Day 4 21.7	30.8 31.5
Skalkidou 2003[310, 311]	Day 0-5	jaundice	209	3.3	Nichols Advantage Automated Specially System (Nichols Institute, San Juan Capistrano, CA)		3.4	24	44.5
Skalkidou 2003[310, 311]	Day 0-5	Non jaundice	123	3.3	Nichols Advantage Automated Specially System (Nichols Institute, San Juan Capistrano, CA)		2.1	29.1	56.2
Elmlinger 2004[312]	Day 1-7		45		Two site, solid phase chemiluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corp, Los Angeles, CA)	IGF-II used to block IGFBP binding sites Assay specific diluent used	3	15	27
Elmlinger 2004[312]	Day 8-14		40		Two site, solid phase chemiluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corp, Los Angeles, CA)	IGF-II used to block IGFBP binding sites Assay specific diluent used	7	25	43
De Zegher 2012[313]	2 weeks	AGA	72	3.9 (3-4.8)	Immunochemiluminescence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA)		0	55	115.6
De Zegher 2012[313]	2 weeks	SGA	102	2.9 (1.9-3.9)	Immunochemiluminescence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA)		0	46	107.8
De Zegher 2012[313]	4 months	AGA	72	7.2 (5.5–8.9)	Immunochemiluminescence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA)		7.7	51	94.3
De Zegher 2012[313]	4 months	SGA	102	6.4 (5.4–7.4)	Immunochemiluminescence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA)		0	69	141.1
Elmlinger 2004[312]	0.5-6 months		25		Two site, solid phase chemiluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corp, Los Angeles, CA)	IGF-II used to block IGFBP binding sites Assay specific diluent used	6	156	306

Table 2.2.4: Assay Specific IGF-I measurement 6-18 months of age

Author	Timing	Population	Number	Weight	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
ELISA									
Yuksel 2011[265]	12 months	Males	54		ELISA (Diagnostic Systems Laboratories Inc)		0	82.1	211.1
Yuksel 2011[265]	12 months	Females	45		ELISA (Diagnostic Systems Laboratories Inc)		0	125.3	310.9
Radioimmunoassay									
Leger 1996[251]	6 months		12		RIA using polyclonal IGF-I antiserum	Acid chromatography	30	102	174
Cassio 1998[248]	7 months		10		Nichols Institute Diagnostics, San Juan Capistrano, CA	Acid ethanol extraction	26.9	83.7	140.5
Barton 1996[314]	12 months		40		Radioimmunoassay. DSL-6600 Diagnostic Systems Laboratories, Texas.		0	61	136.9
Iniguez 2006[296]	12 months	SGA	50		Locally developed radioimmunoassay	With extraction	27.4	67	106.6
Leger 1996[251]	12 months		37		RIA using polyclonal IGF-I antiserum	Acid chromatography	3	73	143
Mamabolo 2007[315]	12 months		116	9.4kg (6.5-12.2)	RIA on the Cobra II Gamma Counter. (Diagnostic Systems Laboratories Inc, Webster, TX, USA)		0	24.8	82.2
Ong 2009[301]	12 months	Breastfed	61 boys 57 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	8 3.6	Boys 38.8 Girls 56	69.6 108.4
Ong 2009[301]	12 months	Formula fed	92 boys 68 girls		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Extracted from filter paper using acidifying buffer	4.8 7.3	Boys 47.6 Girls 59.9	90.4 112.5
Rajaram 1995[295]	12 months		51	Birthweight 3.3 (2.5-4.1)	Radioimmunoassay (Endocrine Sciences, Calabasas Hills, CA)		46.6	56	65.4
Juul 1994[316]	1-2 years		44		Radioimmunoassay (Mediagnost, Tubingen, Germany)	Acid-ethanol extraction and cryoprecipitation.	29	80	157
IRMA									
Low 2001[304]	12 months	Term, AGA	48		Immunoradiometric assay (Diagnostic Systems Laboratories Inc, Webster, TX, USA). 2 site assay.		0	74	151.9
Low 2001[304]	18 months	Term, AGA	48		Immunoradiometric assay (Diagnostic Systems Laboratories Inc, Webster, TX, USA). 2 site assay.		0	104	233.8
Hyun 2012[268]	1-2 years	Males	35		IRMA (Immunotech, Marseilles, France)		0	57.9	123
Hyun 2012[268]	1-2 years	Females	25		IRMA (Immunotech, Marseilles, France)		0	92.4	183.8

Table 2.2.4 (continued)

Author	Timing	Population	Number	Weight	Assay	Extraction	-2SD	Mean (ng/ml)	+2SD
Chemiluminescent immunometric assays									
Larnkjaer 2009[317]	9 months	Formula Feeding	27		Automated chemiluminescent assay (IMMULITE 1000, DPC Biermann GmbH, Bad Nauheim, Germany)	Pretreatment with acid	20.2	58.4	96.6
Elmlinger 2004[312]	6-12months		19		Two site, solid phase chemiluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corp, Los Angeles, CA)	IGF-II used to block IGFBP binding sites Assay specific diluent used	38	140	242
Larnkjaer 2009[317]	12 months	Formula Feeding	28		Automated chemiluminescent assay (IMMULITE 1000, DPC Biermann GmbH, Bad Nauheim, Germany)	Pretreatment with acid	7.8	54	100.2
Brabant 2003[318]	1-2 years		77		Two site chemiluminescent immunoassay (Nichols Advantage; Nichols Institute Diagnostics, Calif, USA)	Acidification Incubated with excess IGF-II and acridium-ester labeled antibody	2	58	114
Elmlinger 2004[312]	1-2 years		27		Two site, solid phase chemiluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corp, Los Angeles, CA)	IGF-II used to block IGFBP binding sites Assay specific diluent used	36	134	232

2.2.4 DISCUSSION

Assay variability in GH and IGF-I measurement can present a significant challenge in the comparison of results between studies and in clinical interpretation. This review highlights the wide variability in reported measurements between studies related both to differences in the study population and in assays used.

The diagnosis of GH deficiency in the paediatric population can be challenging. In the absence of a robust test for diagnosing GH deficiency, paediatric endocrinologists vary in practice[62, 135, 136]. Serum IGF-I levels are stable and random values can be clinically useful in this evaluation, with a sensitivity of 70-90% in the detection of isolated GH deficiency[319]. The GHST is commonly used, but peak GH response to stimulation testing is influenced by assay

methodology[233]. Despite this, almost half of physicians who rely on stimulation testing do not know what type of GH assay is utilised by their laboratory[133].

Clinical, auxological and radiological data can also guide the clinician in diagnosing GH deficiency[8], but these can be less useful in infants and small children. Children with isolated congenital GH deficiency have a normal birth weight and length, and often present only later with growth failure[219]. Hypoglycaemia can be problematic in this population, and may be the only clinical feature[13, 46, 212].

GH and IGF-I measurements currently play a central role in our evaluation of children with possible GH deficiency. In this review, we have highlighted that there is wide variability in reported means and standard deviations for IGF-I and GH concentrations in this age group, and this adds to the difficulty in diagnosing GHD in infants.

2.2.5 Chapter Conclusion

Assay variability and interference can contribute to difficulty in using IGF-I and GH measurements to guide clinical care, and can cause misclassification of results as being abnormal in some cases. This review has demonstrated wide variability in the reported concentrations of IGF-I in this age group. In the following chapters, I will explore a new modality of measuring IGF-I that is less likely to be subject to the interference seen with current assays.

CHAPTER 2.3: MASS SPECTROMETRY

Publication

Hawkes CP, Schnellbacher S, Singh RJ, Levine MA. 25-hydroxyvitamin D can interfere with a common assay for 1,25-dihydroxyvitamin D in vitamin D intoxication. J Clin Endocrinol Metab. 2015;100(8):2883-9 (Appendix G).

In the previous chapter, I have reviewed the reference data for IGF-I concentrations in infancy and demonstrated wide variation across studies. IGFBP interference may contribute significantly to these differences between assays. In this chapter, I will provide an overview of the mechanisms of this interference in competitive and non-competitive assays. I will then explore the potential of mass spectrometry to overcome interference. This will provide the background for the next chapter, where I will describe reference data for IGF-I concentrations in infants using mass spectrometry.

2.3.1 THE EFFECT OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN INTERFERENCE ON INSULIN-LIKE GROWTH FACTOR-I MEASUREMENT

Competitive immunoassays rely upon serum IGF-I competing with a labeled antigen for an antibody (Figure 2.3.1A). Measurement of the antibody-labeled antigen complex allows for measurement of serum IGF-I using this technique. IGFBP binding to IGF-I can interfere with competitive immunoassays in two ways. When bound to an IGFBP, IGF-I may not bind to the antibody and give a falsely low measurement (Figure 2.3.1B). IGFBPs in serum may also bind to the labeled antigen and cause the opposite effect, i.e. result in a falsely high measurement of IGF-I (Figure 2.3.1C).

Non-competitive assays require the binding of labeled antibodies to specific epitopes on the protein of interest (Figure 2.3.2A). The presence of binding proteins bound to IGF-I can prevent epitope recognition, and consequently cause the measurement to be erroneously low (Figure 2.3.2B).

Separation of IGF-I from its binding proteins is necessary to overcome the unreliable measurements caused by IGFBP interference, although it is not generally possible to remove all IGFBPs from solution. As discussed in Chapter 2.2, the most common approach to this is the disassociation of IGF-I from IGFBPs through acidification, followed by separation using gel chromatography or ethanol precipitation[320]. Saturation of binding proteins is often performed by the addition of excess IGF-II or other IGFBP blocking agents.

It is important to note that performing any separation technique results in the measurement of total IGF-I and does not distinguish free from total IGF-I concentrations. The clinical utility of free IGF-I measurement is unknown and total IGF-I is currently considered to be appropriate for clinical use[321, 322].

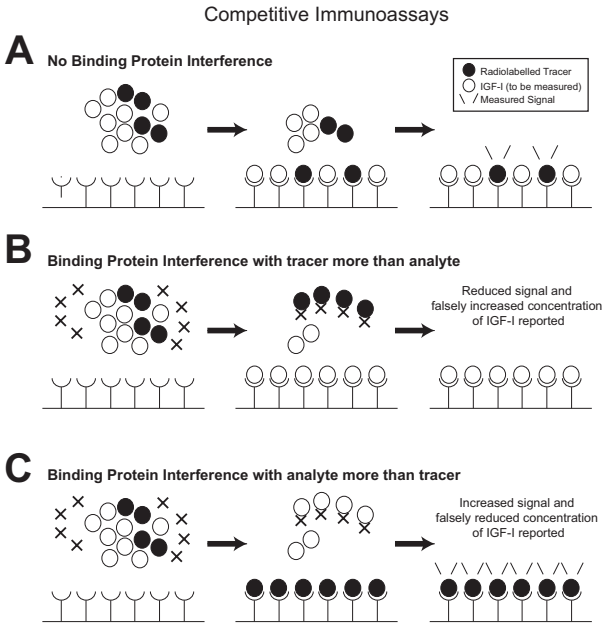

Figure 2.3.1: The principles of competitive immunoassays in IGF-I measurement, and the potential effects of IGFBP interference

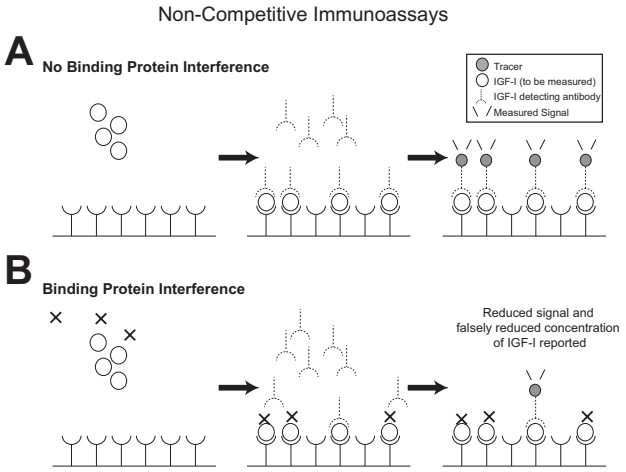

Figure 2.3.2: The principles of non-competitive immunoassays in IGF-I measurement, and the potential effects of IGFBP interference

Numerous competitive and non-competitive IGF-I assays are currently available, and most are calibrated against the WHO 87/518 IGF-I standard[323]. Despite this calibration, differences are seen when assays are compared with each other[260, 324] and it is thought that these differences are due to variations in sensitivity of each assay to interference from binding proteins[323].

The following study highlights the potential of mass spectrometry to overcome interfering substances and provide more accurate measurements than RIAs. This study prompted our move towards studying IGF-I measured by this modality in subsequent chapters.

2.3.2 Mass Spectrometry is Less Sensitive than Immunoassays to Interference

My impetus for exploring the potential of mass spectrometry to overcome interference comes from a study I completed in using this modality in children with vitamin D intoxication (Appendix G)[211]. At the Children's Hospital of Philadelphia, we saw two patients with elevated serum levels of 25-

hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) when measured by RIA.

To become fully active, vitamin D must undergo two modifications by cytochrome P450 (CYP) enzymes. The first step is 25-hydroxylation of parent vitamin D_2 and D_3 by hepatic microsomal CYP2R1, which generates the prohormones 25-hydroxyvitamin D_3 ($25(OH)D_3$) and 25-hydroxyvitamin D_2 ($25(OH)D_2$), respectively[325]. A second hydroxylation occurs in the kidney where CYP27B1, a 1α-hydroxylase located in mitochondrion, converts 25(OH)D to the fully active form of the vitamin, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Figure 2.2.3). $1,25(OH)_2D$ is a potent hormone, and binds with high affinity to the vitamin D receptor (VDR), which mediates most physiological actions of vitamin D via modulation of the transcription of target genes.

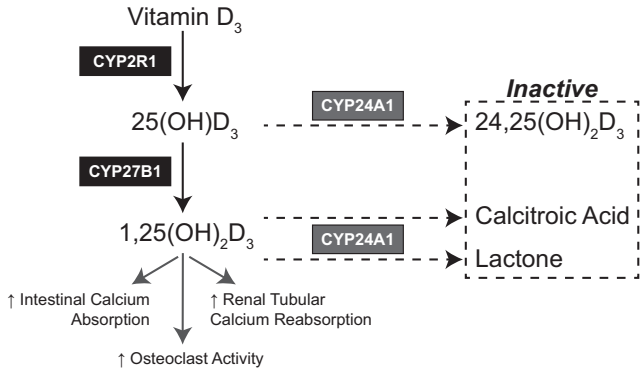

Figure 2.3.3: Overview of Vitamin D metabolism

The conversion of 25(OH)D to $1,25(OH)_2D$ by CYP27B1 is tightly regulated by calcium, parathyroid hormone (PTH) and fibroblast growth factor 23. Hence, as serum levels of 25(OH)D increase excessively there is a parallel decrease in levels of serum $1,25(OH)_2D$, a phenomenon that reflects suppression of PTH by hypercalcaemia and a corresponding loss of PTH-dependent stimulation of CYP27B1 activity[326].

Thus, elevated levels of $1,25(OH)_2D$ on RIA measurement seen in our two patients did not make sense. We subsequently used a LCMS assay to measure

$1,25(OH)_2D$ in these patients and demonstrated that $1,25(OH)_2D$ levels were not elevated (Table 2.3.1).

Table 2.3.1: Clinical characteristics and laboratory findings in two adolescents presenting with 25(OH)D intoxication

	Reference Range	**Case 1**	**Case 2**
Age, Sex		15 years, male	17 years, female
Background Diagnoses		Well child	Ocular Albinism, Autism
Calcium (mg/dL)	8.8 – 10.1	13.8	11.3
Phosphorous (mg/dL)	2.9 – 5.4	4.2	4
Albumin (g/dL)	3.7 – 5.6	4.3	4.1
Parathyroid Hormone (pg/ml)	9 - 52	<3	<5.5
Creatinine (mg/dL)	0.6 - 1.3	3.1	0.48
PTHrP (pmol/L)	<2	<2	<2
CYP24A1 gene		Normal	Normal
$25(OH)D_3$ (ng/ml) LCMS	30 - 80	685	143
$1,25(OH)D_3$ (pg/ml) RIA	15 - 75	>230	>190
$1,25(OH)D_3$ (pg/ml) LCMS	25 – 86	45	68

This experience prompted me to explore the role of LCMS assays in other areas where assay interference may compromise the utility of RIA, notably IGF-I and –II measurement.

2.3.3 CHAPTER CONCLUSION

The majority of assays currently used to measure IGF-I concentrations are RIAs. Competitive and non-competitive immunoassays are subject to unpredictable interference by IGFBPs when used to measure IGF-I concentration. In this chapter, I have described my experience of using LCMS to overcome interference of 25(OH)D in the measurement of $1,25(OH)_2D$ with RIA. In the following chapter, I will explore the potential of a novel LCMS assay in measuring IGF-I and –II concentrations.

CHAPTER 2.4: CORRELATION OF INSULIN-LIKE GROWTH FACTOR-I AND –II CONCENTRATIONS MEASURED BY MASS SPECTROMETRY AT BIRTH WITH GROWTH FROM BIRTH TO TWO MONTHS

Publication

Hawkes CP, Murray DM, Kenny LC, Kiely M, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Correlation of insulin-like growth factor-I and –II concentrations at birth measured by mass spectrometry and growth from birth to two months. Horm Res Paediatr. 2018 Jan. doi 10.1159/000486035 [Epub ahead of print] (Appendix H).

Presentation

Hawkes CP, Murray DM, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Measurement of IGF-I and –II concentrations at birth by mass spectrometry in a large birth cohort: correlation with anthropometry. Pediatric Endocrine Society, September 2017 (Poster) (Appendix I).

In the preceding chapters, I have described wide variability in GH and IGF-I concentrations in infancy and summarised the mechanisms for interfering substances in affecting these results. At birth, IGF-I concentrations are low and serum IGFBP concentrations vary. Consequently, if clinically significant assay interference occurs, newborn infant IGF-I measurement would be the age group with the most imprecise measurements.

2.4.1 BACKGROUND

IGF-I and –II play an important role in prenatal growth, as evidenced by mutations affecting IGF-I[152, 327] and IGF-II[328, 329] signaling significantly reducing birth size. The magnitude of this effect is up to 60% in mouse models[330, 331]. Importantly, congenital GHD is associated with normal size at birth[332], demonstrating that prenatal IGF-I and –II regulation is less dependent on GH production[333] than in the older child.[334]

Numerous factors outside of the GH/IGF axis may influence prenatal IGF-I and –II production. Umbilical cord blood samples from female infants have higher IGF-I concentrations than from males [335]. Maternal factors such as cigarette smoking[336], obesity[337], and preeclampsia[279, 338] may also affect IGF concentrations, although most studies to date have focused on IGF-I and not IGF-II measurements. Furthermore, small studies have demonstrated a correlation between cord IGF-I concentration at birth and birth size[221, 262, 280], but the association for IGF-II is reported to be weak or absent[221, 277, 339].

Over 90% of circulating IGF-I and -II is bound to IGFBPs, prolonging their serum half-life and regulating their bioavailability. Prior to measurement, IGF-I and –II must be separated from these IGFBPs, and extraction methods such as acid ethanol precipitation are commonly used [320]. This approach recovers only 75-80% of the IGF-I complexed to IGFBPs[236], and may affect the reliability of the reported measurements. This interference is likely to be more problematic in infants, who have lower IGF concentrations and higher concentrations of some of the IGFBPs than older children[124]. For example, IGFBP-1 concentrations can be increased by the stress of labour[340, 341], or in pregnancies complicated by pre-eclampsia[279], and it is likely that interference will significantly affect IGF measurement accuracy when susceptible assays are used. Differences in IGF-I measurements also have been attributed to their reliance on different reference populations, resulting in assay-specific reference ranges. A study of six IGF-I immunoassays using the same healthy population-based reference samples still yielded different assay-specific reference ranges with variations most noticeable at higher IGF-I concentrations[260].

LCMS assays are now available to measure IGF-I and –II concentrations[342], a method that is less susceptible to IGFBP interference even in the presence of supra-physiological levels of IGFBP-3[238]. Isotopically labeled IGF-I can be used with the LCMS assay to adjust for the IGF-I and –II lost in acid ethanol extraction and chromatography, further improving the accuracy of this method.

2.4.2 AIM

The aim of this study was to explore the relationships of age- and gestational age-corrected umbilical cord IGF-I and –II concentrations measured at birth by LCMS with weight, length, and occipitofrontal head circumference (OFC) at birth and two months. As a secondary objective, we aim to describe gestational age- and sex-specific reference data for IGF-I and IGF-II concentrations at birth using an LCMS assay.

2.4.3 METHODS

2.4.3.1 The SCOPE Pregnancy Study and Cork BASELINE Study

The SCOPE pregnancy study[343] is a multicentre cohort study that recruits primiparous, low risk women at 15±1 weeks' gestation. The aim of the SCOPE study is to develop biomarkers for the prediction of pre-eclampsia, fetal growth restriction and pre-term birth in a low risk population. Therefore the specific exclusion criteria were: multiple pregnancies, known major fetal anomalies, pre pregnancy essential hypertension, diabetes, renal disease, systemic lupus erythematous, antiphospholipid syndrome, major uterine anomaly, cervical cone biopsy, ≥3 miscarriages and treatment with low dose aspirin, calcium intake >1g/24 hours, low molecular weight heparin, fish oil and antioxidants.

The Cork BASELINE (Babies After Scope; Establishing the Longitudinal Impact using Neurological and nutritional Endpoints) study is a longitudinal birth cohort study established as a follow on to the SCOPE pregnancy study in Ireland. Women recruited to the SCOPE Ireland study were approached at 20 weeks' gestation and recruited to the BASELINE study. In addition, firstborn healthy children born between August 2008 and August 2011 were eligible for this study. A detailed description of this cohort has been reported previously[344]. The Clinical Research Ethics Committee of the Cork Teaching Hospitals approved this study.

2.4.3.2 Subjects

For this study, all children born between 37 and 42 weeks gestation were included. Gestational age was determined by the first day of the last menstrual period. If fetal ultrasound was performed before 16 weeks' gestation and a discrepency of greater than 6 days was noted, ultrasonographic gestational age was used. Similarly, gestational age was determined by ultrasound if performed up to 21 weeks' gestation and a discrepency of 10 days was noted. Smoking status during the first trimester was ascertained by self-report during pregnancy.

2.4.3.3 Anthropometry

Weight, length, and OFC were measured on the first day of life using standardised protocols. A Harpenden neonatometer (Harlow Healthcare, London, UK) was used to measure supine length, and naked weight was measured using the Seca 384 Baby Scales (Seca, Hamburg, Germany). Sex- and gestational age-specific z-scores for weight, length, and OFC were generated from the United Kingdom reference charts[345] using Stata 12.0 (StataCorp, College Station, TX, USA)[346]. Subjects were measured again at two months of age using the same standardised protocols.

2.4.3.4 Sample collection and storage

Umbilical cord blood samples were collected at birth and processed to serum within three hours of collection. They were stored at -80^{o}C until analysis.

2.4.3.5 Mass Spectrometry

IGF-I and –II concentrations were measured by Quest Diagnostics (San Juan Capistrano, CA, USA) using LCMS[238, 241]. Isotopically labeled IGF-I was added to the sample as an internal standard for IGF-I and -II. IGF-I and -II were released from their binding proteins by an acid ethanol extraction followed by automated online extraction and analytical chromatography using an Aria TX-4 (Thermo-Fisher, San Jose, CA, USA). IGF-I and -II were quantitated using a time-of-flight mass spectrometer using narrow mass extraction of full-scan

spectra. The internal standard was used to adjust for any procedural losses during extraction.

Performance characteristics of this assay have been previously described[238, 241]. For IGF-I using quality control samples the inter-assay coefficient of variation and percent recovery were 5% and 104% at 100 ng/ml, 5.2% and 103% at 400 ng/ml and 3.5% and 103% at 700 ng/ml. For IGF-II, the inter-assay coefficient of variation and percent recovery were 6.1% and 102% at 200 ng/ml, 3.2% and 99% at 500 ng/ml and 5.3% and 99% at 1200 ng/ml. Similar performance was seen with serum pools[238, 241].

2.4.3.6 Statistical Analysis

The lower limit of detection of IGF-I using this assay is 16 ng/ml; samples with concentrations below this limit were assigned a value of 15 ng/ml. Similarly, the lower limit of detection of IGF-II was 32 ng/ml; samples with concentrations below 32 ng/ml were assigned a value of 31 ng/ml.

Sex-specific reference curves for IGF-I and -II concentrations were generated using LMS Chartmaker Pro (Harlow Printing Ltd., Tyne and Wear, UK). The LMS method[347, 348] uses a Box-Cox transformation to obtain normality. Three distinct curves were generated for skewness, median, and variability. These were combined in one graph, with smoothening of changes over time. They were then adjusted until fit of the curve was visually optimised as per software recommendations. IGF-I and -II concentrations were then converted to age- and sex-specific z-scores from this population for subsequent analysis.

Data analyses were performed using SPSS 22.0 (IBM, New York, NY, USA). Normally distributed data were described as mean (SD) and compared using independent-sample t-tests. Linear regression analysis was used to determine the relationship between continuous variables.

2.4.4 RESULTS

Eleven hundred term infants (563 male) met the inclusion criteria for this study. Characteristics of the population are shown in Table 2.4.1.

Table 2.4.1: Characteristics of the infants and pregnancies included in this cohort. Values of all continuous variables are presented as mean (SD).

	Male	**Female**	**All**
Number of infants, n	563	537	1100
Gestational age, weeks	40.1 (1.1)	40.2 (1.1)	40.2 (1.1)
Weight, kg	3.55 (0.47)	3.48 (0.44)	3.52 (0.45)
Length, cm	50.7 (2.1)	50.2 (1.9)	50.4 (2)
Head circumference, cm	35.1 (1.4)	34.6 (1.3)	34.9 (1.4)
Maternal age, years	30.1 (4.6)	30 (4.2)	30.1 (4.4)
Maternal BMI at 15 weeks, kg/m^2	24.8 (4.1)	24.9 (4.2)	24.9 (4.1)
Caucasian, n	555	527	1082
Maternal smoking during first trimester, n	145	132	277
IGF-I, ng/ml	48.5 (23.2)	56.7 (23.9)	52.5 (23.9)
IGF-II, ng/ml	420.8 (95)	428 (101.4)	424.3 (98.2)

The mean (SD) IGF-I concentration was 52.5 (23.9) ng/ml (males 48.5 [23.3] ng/ml, females 56.7 [23.9] ng/ml). The mean (SD) IGF-II concentration was 424.3 (98.2) ng/ml (males 420.8 [95] ng/ml, females 428 [101.4] ng/ml). There was a significant difference between males and females in IGF-I ($p < 0.001$) but not in IGF-II concentrations ($p = 0.2$).

Linear regression analysis showed a slight reduction in IGF-I concentrations with increasing gestational age in both males (regression coefficient [SEM] -0.005 [0.002], $p = 0.01$) and females (regression coefficient [SEM] -0.004 [0.002], $p = 0.03$). Gestational age was not associated with IGF-II concentrations in either males (regression coefficient [SEM] 0 [0], $p = 0.4$) or females (regression coefficient [SEM] 0.001 [0], $p = 0.06$). IGF-I and –II concentrations

in term male and female infants according to gestational age are presented in Figure 2.4.1.

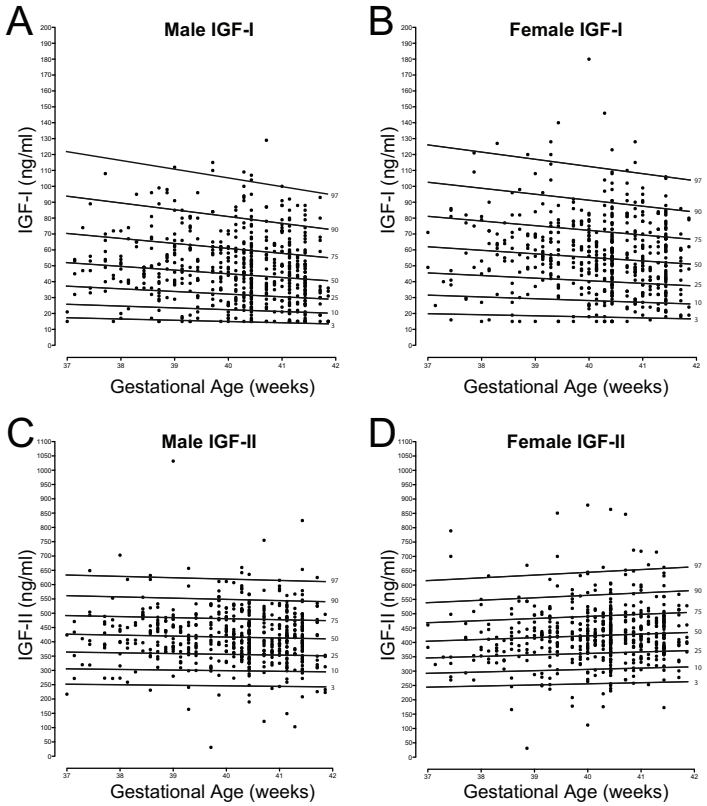

Figure 2.4.1: Sex-specific centile charts for IGF-I (A and B) and IGF–II (C and D) concentrations in term infants in a healthy population-based cohort, according to gestational age from 37 to 42 weeks' gestation.

Correlation with Anthropometry and Early Growth

Z-Scores for gestational age- and sex-specific IGF-I concentrations at birth correlated with weight, length, and OFC z-scores at birth. The strongest association was with birth weight (R^2=0.19). Length and weight z-scores at age two months correlated significantly with IGF-I concentrations at birth, but the strength of the association was lower than at birth. Over two months, weight R^2 decreased from 0.19 to 0.02 and length R^2 decreased from 0.07 to 0.04. Although IGF-II concentrations correlated significantly with weight, length, and OFC at birth, this accounted for <1% of the variance seen in each of these parameters (Table 2.4.2).

Both IGF-I and -II concentrations at birth were associated with length at age two months ($p < 0.001$ and $p = 0.04$ respectively), while only IGF-I concentration at birth was associated with weight at two months ($p < 0.001$). The rates of change in OFC and weight z-scores over the first two months of life were negatively associated with IGF-I concentrations at birth. IGF-II concentrations at birth were not associated with growth trajectories in weight, length, or OFC over the first two months (Figure 2.4.2).

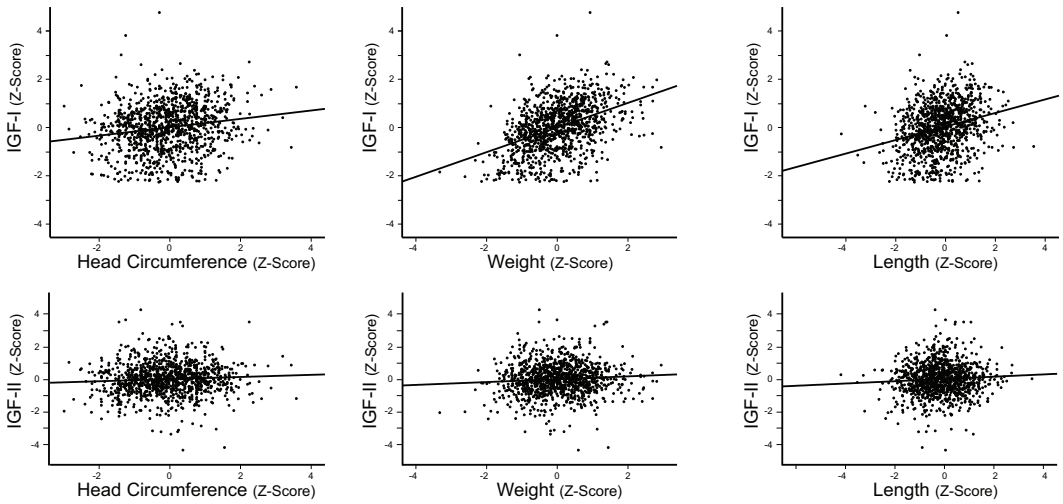

Figure 2.4.2: The relationship between IGF-I and –II z-scores at birth with head circumference, weight and length z-scores at birth.

An increase in weight, OFC, and length z-score of 1 or more over the first two months was seen in 198, 250, and 343 infants respectively. Those with an increase in weight z-score of 1 or more had significantly lower mean IGF-I z-scores at birth (-0.6 vs 0.1, $p<0.001$), but not IGF-II z-scores (-0.06 vs 0.02, $p = 0.4$). Similarly, infants with an increase in OFC z-score during the first two months tended to have lower IGF-I z-scores (-0.2 vs 0.05, $p = 0.003$) but not IGF-II z-scores (-0.08 vs 0.02, $p = 0.2$). No difference was seen in IGF-I ($p = 0.9$) or IGF-II ($p = 0.9$) z-scores at birth in infants who had an increase in length z-score of 1 or more. Lower mean (SD) birth weight z-scores were seen in infants with increases in z-scores of 1 or more in the first two months for weight

(-0.5 vs 0.1, p < 0.001), length (-0.08 vs 0.09, p < 0.001) and OFC (-0.3 vs 0.1, p < 0.001).

Table 2.4.2: The relationship between sex- and gestational age-corrected IGF-I and IGF-II z-scores and growth parameters at birth and 2 months, and growth trajectory from birth to 2 months.
Length, weight and head circumference are presented as sex-, age- and gestation corrected z-scores. For this analysis, IGF-I and –II are the dependent variables and anthropometric measures are independent variables.

	IGF-I			IGF-II		
	R^2	Regression coefficient (SEM)	p	R^2	Regression coefficient (SEM)	p
Z-Scores at Birth						
Length	0.07	0.28 (0.03)	<0.001	0.004	0.07 (0.03)	0.03
Weight	0.19	0.51 (0.03)	<0.001	0.005	0.09 (0.04)	0.01
Head Circumference	0.028	0.17 (0.03)	<0.001	0.004	0.07 (0.03)	0.04
Z-Scores at 2 months						
Length	0.04	0.21 (0.03)	<0.001	0.004	0.07 (0.03)	0.04
Weight	0.022	0.15 (0.03)	<0.001	0.002	0.05 (0.03)	0.16
Head Circumference	0.001	0.04 (0.04)	0.24	0.003	0.06 (0.04)	0.06
Delta z-score from birth to 2 months						
Length	0.002	-0.05 (0.04)	0.14	0.001	0.01 (0.04)	0.8
Weight	0.07	-0.31 (0.04)	<0.001	0.001	-0.03 (0.04)	0.4
Head Circumference	0.02	-0.17 (0.04)	<0.001	0.001	-0.02 (0.04)	0.5

Factors influencing IGF concentrations

Of the 1100 infants included in this study, 40 had birthlength and 57 had birthweight below the 10th percentile. In those with birthlength below the 10th percentile, IGF-I z-scores (-0.7 vs 0.1, p <0.001) and IGF-II z-scores (-0.3 vs 0, p 0.03) at birth were lower (Figure 3D). This was also seen in infants with birthweight below the 10th percentile (IGF-I z-scores -1.2 vs 0.07, p < 0.001; IGF-II z-scores -0.4 vs 0, p = 0.01) (Figure 2.4.3).

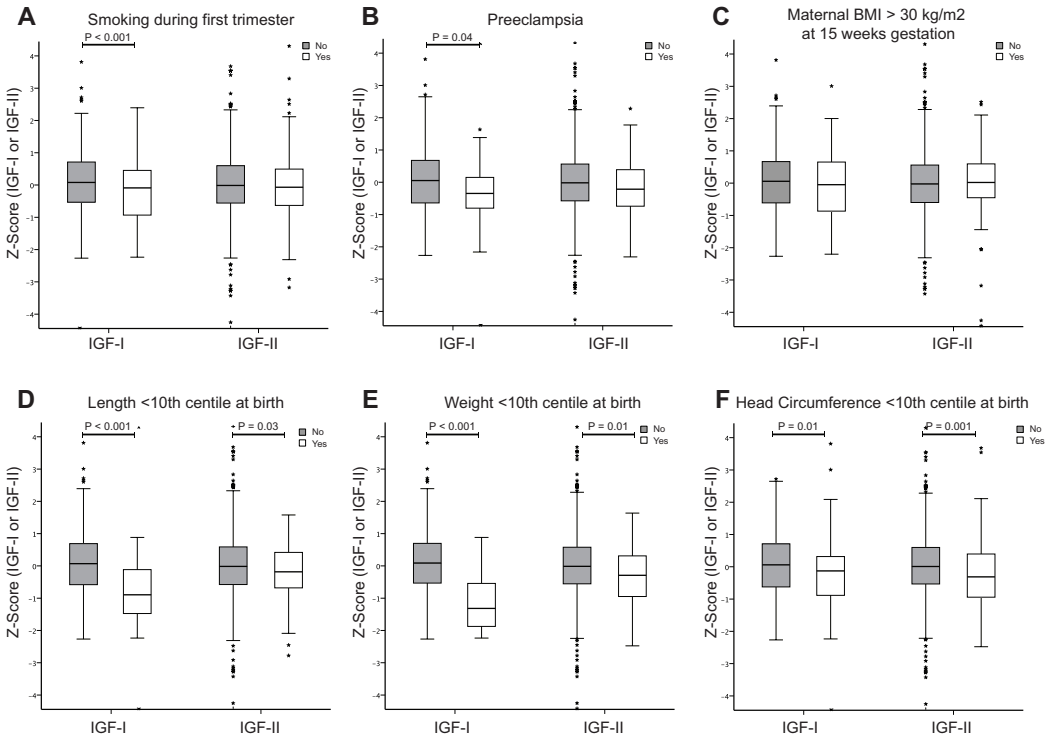

Figure 2.4.3: The effect of smoking (A), preeclampsia (B) and maternal obesity (C) on umbilical cord IGF-I and –II age- and-sex specific z-scores. The association of birth length (D), birth weight (E) and birth length and head circumference (F) below the 10th centile with cord IGF-I and –II concentrations are also shown.

Maternal cigarette smoking during the first trimester and pre-eclampsia were associated with lower mean (SD) cord IGF-I concentrations ($p < 0.001$ and $p = 0.04$ respectively) but not IGF-II concentrations ($p=0.2$ and $p=0.5$ respectively). Maternal BMI > 30 kg/m^2 (n = 137) at 15 weeks' gestation was not associated with increased umbilical cord IGF-I or –II concentrations ($p = 0.4$ and 0.5 respectively) (Table 2.4.3).

Table 2.4.3: Comparison of gestation- and sex-corrected mean (SD) z-scores for IGF-I and –II concentrations at birth, according to maternal smoking, maternal obesity, pre-eclampsia, and length, weight and OFC percentiles.

	IGF-I z-score			IGF-II z-score		
	Yes	No	p	Yes	No	p
Smoking during first trimester	-0.2 (1) n=277	0.07 (0.99) n=823	<0.001	-0.07 (1) n=277	0.01 (1.04) n=823	0.2
Maternal BMI > 30 kg/m^2 at 15 weeks' gestation	-0.07 (1.1) n=137	0.01 (0.99) n=963	0.4	0.05 (1.2) n=137	-0.02 (1) n=963	0.5
Pre-eclampsia	-0.31 (0.85) n=34	0.01 (1) n=1066	0.04	-0.13 (1.01) n=34	-0.01 (0.01) n=1066	0.5
Birthlength <10th percentile	-0.79 (0.87) n=48	0.04 (1) n=1052	<0.001	-0.33 (0.99) n=48	0 (1.02) n=1052	0.03
Birthweight <10th percentile	-1.17 (0.77) n=59	0.07 (0.97) n=1041	<0.001	-0.35 (0.94) n=59	0.01 (1.03) n=1041	0.01
OFC <10th percentile	-0.21 (1.01) n=139	0.03 (1) n=961	0.01	-0.31 (1.2) n=139	0.03 (1) n=961	0.001

2.4.5 DISCUSSION

We have described IGF-I and –II concentrations at birth using a LCMS assay[238], thus generating accurate measurements at an age where concentrations tend to be low. Our large cohort of well-characterised term infants allowed us to investigate the relationship between these measurements and birth size and early infant growth. Consistent with previous studies using other assays, IGF-I and –II concentrations at birth were associated with weight, length, and OFC at birth. At age two months, weight and length were associated with birth IGF-I concentrations whereas only length was associated with birth IGF-II concentrations. Low IGF-I concentrations at birth were associated with accelerated increases in weight and OFC z-scores over the first two months. Maternal smoking and pre-eclampsia were associated with lower cord IGF-I but not IGF-II concentrations.

The correlation of an infant's birth weight and length with IGF-I concentration has been shown previously in smaller studies using RIAs[221, 277, 278, 336, 349]. The relationship between birth weight and IGF-II levels are less well defined, with

some studies showing no correlation[349, 350]. Our results support the findings of other studies, where a weak association has been shown[221, 351]. This weak association between cord IGF-II and neonatal anthropometry is surprising given the significant effect of mutations affecting IGF-II signaling on mouse[330] and human[328, 329] size at birth. Possible explanations for this include a potential neutralising effect of circulating IGF-II receptors on the bioavailability of circulating IGF-II[221]. This interference may be of clinical significance but not affect IGF-II measurement by LCMS. Alternatively, IGF-II may be more important in regulating growth prior to the third trimester[352, 353], and IGF-II concentrations at birth may be clinically less significant.

Infants with lower IGF-I concentrations at birth had a greater change in weight and OFC over the first two months of life. The mechanism of this effect is unclear, but this finding may be related to low IGF-I concentrations being a marker of nutritional status[196]. Thus, this may represent "catch-up" growth in infants born small for gestational age who initially have lower IGF-I concentrations. An inverse relationship is also seen between early growth and cord leptin, another marker of nutritional status at birth[354]. Birth IGF-II concentrations do not correlate with early infant growth in any of these parameters, further supporting the interpretation that the role of IGF-II in fetal growth is less clinically significant by the time of birth.

Maternal smoking has been shown previously to be associated with reduced cord IGF-I concentrations in females[336]. However, we have shown that this effect is seen both in male and female infants. The interplay between IGF-I and –II and placental function may provide insight into this relationship. Placental vasculature is affected by maternal smoking, with reduced length of villous capillaries and decreased trophoblast volume[355]. This may affect nutrient availability to the fetus and subsequently reduce growth parameters and IGF-I concentrations. IGF-I and –II are both expressed in chorionic and basal plates of the placenta[356], and thus may play a key role in regulating placentation.

The main strengths of this study in evaluating the relationship between IGFs and infant size are the large number of healthy term infants included, and the use of an LCMS assay. This assay has been shown previously to be free from IGFBP interference when measuring IGF-I and IGF-II concentrations[238]. A limitation of this study is that the Cork BASELINE birth cohort was a relatively homogenous population of Caucasian Irish healthy term infants. Consequently, it is not known if the reference data can be applied directly to other patient populations. This may be particularly important when considering the significant effect of smoking during the first trimester on cord IGF-I concentrations, as maternal smoking was seen in a quarter of included pregnancies. IGF-I concentrations may be marginally higher in populations with lower rates of cigarette smoking, such as America where 13% of all females smoke[357]. In addition, our findings are only applicable to term infants and it is not known if the associations are also seen in preterm infants. Not all infants in this cohort had cord blood available for analysis and it is not known if this introduced bias to our study, although this is unlikely as our findings are generally consistent with previous smaller studies. A small number of subjects had IGF-I and –II concentrations below the detectable limits of the assay, and this may also have influenced our results.

In conclusion, this study has provided reference data for IGF-I and IGF-II concentrations at birth in term infants, as measured by LCMS. These data will be useful clinically where prenatal defects of IGF production are suspected, and may support future research in evaluating the prenatal role of these growth factors. We recommend using the LCMS assay when measuring IGF-I and -II in conditions where IGFBP interference may significantly affect interpretation of results, particularly in young infants where IGF concentrations are generally low[124]. Using this assay, we have corroborated many of the previously known associations between IGF-I concentrations and size at birth, demonstrated a weaker but significant association between IGF-II and size at birth and shown a negative association between IGF-I measurements at birth and change in weight or OFC over the first two months of life.

2.4.6 CHAPTER CONCLUSION

The measurement of IGF-I and, to a lesser extent, IGF-II can give insight into GH production and action and impaired IGF production can affect growth. In Section 1, I highlighted challenges in demonstrating sufficient GH production through stimulation testing. Here, I have focused on imperfections in IGF-I measurement and explored the potential of LCMS to improve accuracy. Interestingly, I have not demonstrated significant improvement in correlating IGF-I concentrations with prenatal growth than have been previously described with RIA. Thus, it is possible that under normal circumstances, IGF-I measurement by LCMS does not provide an advantage over RIA. However, proceeding to LCMS may be a reasonable next step in evaluating the GH/IGF-I axis where RIA measurement is suspected to be incorrect.

SECTION 3

GENETIC APPROACHES TO DISORDERS OF THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS

CHAPTER 3.1: INTRODUCTION

Sections 1 and 2 of this thesis focused on the clinical aspects of evaluating children with short stature. The focus was on optimising measurement of GH secretory capacity and serum IGF-I concentrations, with a view to improving the diagnostic evaluation of children with suspected GHD. In this section, I will focus on another clinical aspect of childhood growth and the GH/IGF-I axis. Specifically, I will describe studies aimed to identify and investigate novel genetic disorders of GH/IGF-I production and signaling.

Monogenic causes of isolated GHD, multiple pituitary hormone deficiencies and disordered GH/IGF-I signaling have been described[358], but many genetic aetiologies of short stature remain to be discovered. Complicating the search for pathogenic mutations and novel of mechanisms of disease is the high prevalence of polymorphisms of unknown or limited clinical significance. Utilisation of established genomic databases, characterised cohorts with described genetic data, and inter-institutional collaborations are often required to establish the significance of previously unknown mutations. In addition to population and clinical data, laboratory-based functional evaluation of the *in vitro* relevance of new mutations is also critical.

I will begin this section by providing an overview of the GH/IGF-I signaling pathway. I will then describe two studies in which we have tried to identify novel mechanisms of disease. Note that, given the large-scale nature of genetic studies, multi-centre collaborations were involved in both studies. Where relevant, I will focus on my specific contribution while giving an overview of the collaborative work.

3.1.1 GROWTH HORMONE SIGNALING

The Growth Hormone Receptor (GHR) is a homodimeric transmembrane protein that is classified as a type 1 cytokine receptor. This consists of an extracellular

domain (ECD) connected to a helical transmembrane domain and an intracellular domain, which contains Box 1 and Box 2 motifs. The extracellular domain contains 267 amino-acids and is encoded by exons 2-7 of the GHR gene. The transmembrane domain contains 24 amino acids and is encoded by exon 8 and the intracellular domain contains 350 amino acids and is also encoded by exon 8.

It was previously thought that GH binding activated the GHR through dimerisation of GHR monomers on the cell membrane. However, it has been shown that GHR dimers exist on the cell surface in the absence of GH binding[359]. One GHR monomer binds with strong affinity to a site on the GH molecule, while the other monomer binds more weakly. This results in relative rotation of one of these monomers[359], leading to repositioning of the intracellular domains and association of the intracellular Box 1 and Box 2 motifs with Janus Kinase (JAK) 2[360] (Figure 3.1.1).

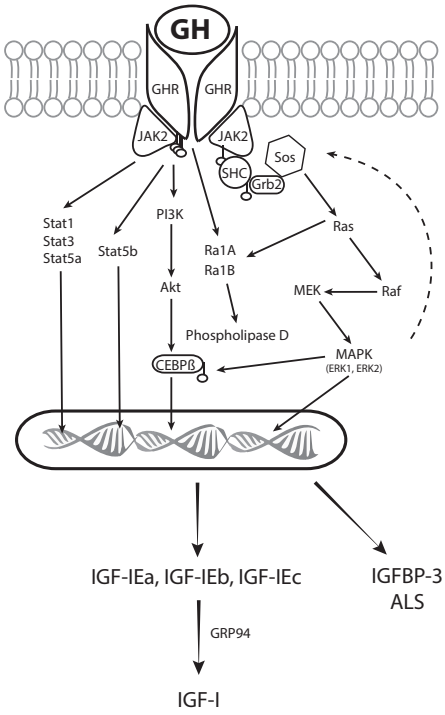

Figure 3.1.1: Growth hormone intracellular signaling

The intracellular domain does not have tyrosine kinase activity, but can bind to JAK2. JAK2 phosphorylates tyrosine residues on the intracellular domain of the

GHR. Phosphorylation of at least three of the seven tyrosines on the intracellular domain of the GHR (Y516, Y548, Y609) is essential for Signal Transducer and Activator of Transcription (STAT) 5b signaling[361]. In addition to its kinase role in initiating the signaling pathway, JAK2 is also important for GHR stability and it reduces endosomal and lysosomal degradation of the GHR[362]. GH predominantly acts through phosphorylation of JAK2, but does also cause phosphorylation of JAK1[363] and JAK3[364]. The phosphorylated intracellular GHR and JAK2 are docking sites for intracellular signaling molecules.

Signal Transducer and Activator of Transcription (STAT) 5b Pathway

The phosphorylated intracellular tyrosines on the GHR recruit STAT5b, which docks via its Src Homology (SH)2 domain. This tyrosyl-phospho-STAT5b complex subsequently forms a homodimer and translocates to the nucleus, where STAT5b-dependent genes are expressed. These include IGF-I, IGFBP3, and ALS. Non-phosphorylated STAT proteins also exist as dimers and can be transcriptionally active at different genes to those targeted by phosphorylated forms[365].

STAT1 and STAT3

STAT1 and STAT3 pathways are both initiated by GH binding to the GHR, independent of the critical tyrosine residues required for STAT5b signaling[361, 366]. The specific roles of these pathways in growth are not clear, and they appear to play a more important role in immune function[367-369].

Ras - Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

In addition to phosphorylating itself and the intracellular component of the GHR, JAK2 also initiates signaling pathways that involve activation of other tyrosine kinases.

Phosphorylated tyrosine residues on JAK2 and/or the intracellular GHR activate the MAPK signaling pathway through initial binding and tyrosyl

phosphorylation of SHC. This then associates with Grb2[370]. SOS is then recruited to the cell membrane and this has guanine nucleotide exchange activity, which activates Ras by initiating guanosine triphosphate (GTP) binding. Once activated, Ras activates Raf, which has serine and threonine kinase activity. This phosphorylates MAPK/ERK Kinase (MEK), which phosphorylates Extracellular Signal Regulated Kinases (ERK1 and ERK2)[371], also known as the MAPK p44/42 pathway. This Raf-MEK-ERK pathway also has negative feedback on SOS through phosphorylation. This causes JAK2 to dissociate from SHC-Grb2, and terminates the signaling pathway.

ERK1 and 2 phosphorylate CEBPβ, a transcription factor that translocates to the nucleus and localises to areas of pericentric heterochromatin and plays a role in regulating MAPK dependent transcription[372, 373]. Although ERKs can enter the nucleus via nucleosomes in an energy-independent fashion, phosphorylated ERKs enter the nucleus at an increased rate through energy dependent mechanisms[374]. Here, they play a role in cell adhesion, migration, survival and differentiation. They also modulate metabolism, and transcription[374].

GH also stimulates the binding of Ras-related protein (Ral)A and RalB to GTP both through JAK2 dependent (via activated Ras) and independent pathways[375]. This increases phospholipase D activity and increases phosphatidic acid formation, which then activates MAPK. GH binding to the GHR also activates the Protein Kinase B (AKT) pathway via the phosphoinositide-3-kinase (PI3K) pathway[376]. This also activates CEBPβ, through release of inhibition from glycogen synthase kinase 3 beta[377].

3.1.2 INSULIN-LIKE GROWTH FACTOR-I PROCESSING AND SIGNALING

IGF-I processing

Alternative splicing of the six exons of the *IGF1* gene on chromosome 12, as well as post-transcriptional modification, result in the production of various IGF-I isoforms. These isoforms differ in the length of their amino terminal polypeptides, and the composition of their extension polypeptides on the carboxy terminal (E-peptides: IGF-IEa, IGF-IEb, and IGF-IEc). Post-translational modification includes cleavage of these E-peptides[378]. The differential roles of each of these IGF-I isoforms are not well understood, but thought to modulate the different biologic effects of IGF-I. These include cell proliferation, differentiation, migration and survival[379].

Glucose-regulated protein 94 (GRP94), a chaperone protein located in the endoplasmic reticulum, plays a key role in the post-translational modification of IGF-I[380]. This has been shown to be critical for the local production of muscle IGF-I. In the mouse model, there is a significant reduction in muscle mass and circulating IGF-I in the presence of depleted muscle GRP-94[381].

IGF Receptors

IGF-I signaling is mediated by binding to the IGF-I receptor. The type 1 IGF receptor (IGF-IR) is a heterotetramer comprising two extracellular α and two transmembrane β subunits. It has approximately 50% structural similarity to the insulin receptor (IR), and this is as high as 84% in the tyrosine kinase domain[382]. Hybrid receptors comprising an IGF-IR αβ heterodimer and an IR αβ heterodimer have also been described[383]. The type 2 IGF receptor (IGF2R) is a monomeric glycoprotein receptor. Unlike the IGF1R, this does not have a signaling domain but undergoes endocytosis upon binding[384]. The IGF1R has greatest affinity for IGF-I and the IGF2R for IGF-II. However, the IR, IGF1R and IGF2R can each bind IGF-I, IGF-II and insulin with variable affinities. As this Section of my thesis will include investigations of mechanisms of IGF-I resistance, I will focus on signaling via the IGF-IR.

IGF Signaling via IGF-IR

IGF-IR is a tyrosine kinase receptor, and IGF-I binding results in autophosphorylation of the receptor, as well as phosphorylation of insulin receptor substrate (IRS) proteins and Shc. Phorsphorylated IRS-1 associates with PI3K, Grb2, Syp, and Nck. Shc can also associate with Grb2. Activated Grb2 associates with Sos, an exchange factor that can activate the Ras-MEK-ERK pathways. These protein kinases, as well as PI3K, and the mechanistic target of rapamycin (mTOR) pathways mediate the downstream effects of IGF-I, including phosphorylation of transcription factors and gene expression[385, 386] (Figure 3.1.2).

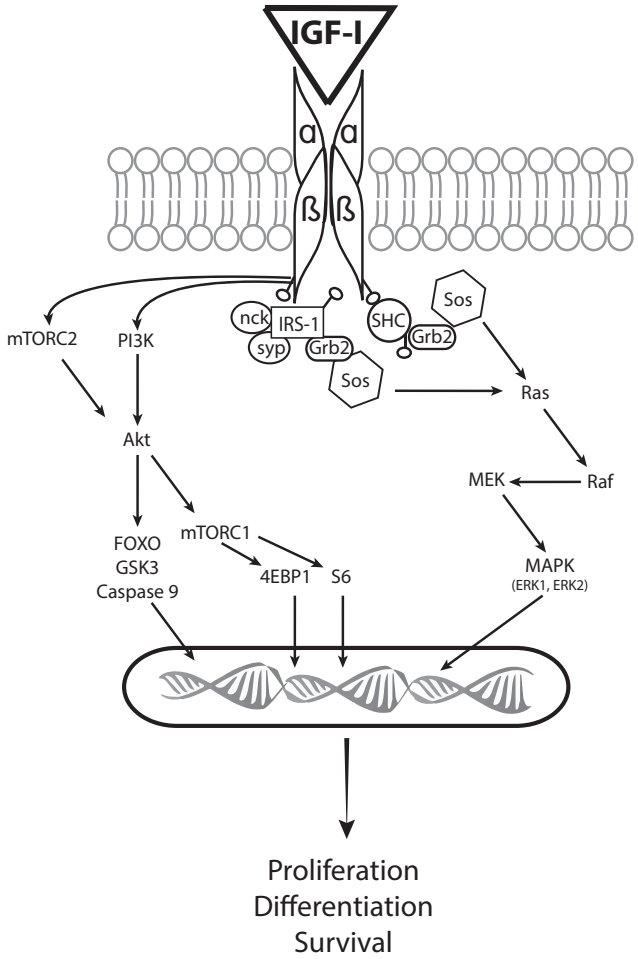

Figure 3.1.2: IGF-I intracellular signaling

CHAPTER 3.2: THE ROLE OF GLUCOSE REGULATED PROTEIN 94 IN INSULIN-LIKE GROWTH FACTOR-I PROCESSING

Publication

Marzec M, Hawkes CP, Eletto D, Boyle S, Rosenfeld R, Hwa V, et al. A human variant of glucose-regulated protein 94 that inefficiently supports IGF production. Endocrinology. 2016;157(5):1914-28 (Appendix J).

IGF-I is produced as a pro-hormone that is subsequently processed by proteases to create the mature, functional hormone. Only processed IGFs are competent to bind to the receptor and protect cells from apoptosis[387], or instruct tissue development *in vivo*[388]. Pro-IGF-I and –II both associate with GRP94 and require its activity before they are processed[387-389].

In this chapter, I describe a multicentre study focused on identifying the *in vitro* and *in vivo* effects of a hypomorphic mutation in *GRP94*, namely a proline substitution for lysine at amino acid 300 (P300L). My role in this study was in the analysis of the clinical phenotype of subjects with this mutation, and will be the focus of this chapter. Although I did contribute to the laboratory-based experiments, this component of the research was not designed by me and is only presented in this thesis as additional background information.

3.2.1 BACKGROUND

GRP94 is a chaperone protein that resides in the endoplasmic reticulum and is selective towards its client proteins. These include immunoglobulins[390], β1-integrins[391], glycoprotein Ib-IX-V complex[392], Toll-like receptors 2, 4, 5, 7, and 9[393], and IGF-I and –II[387, 388].

Although many of the specific interactions of GRP94 with its clients are not fully understood, its function as a chaperone is likely mediated by its ability to bind nucleotides, facilitate ATP hydrolysis, bind calcium and peptides, and undergo conformational changes including dimerisation of its N-Terminal domain[380]. GRP94 exists primarily in an extended conformation in solution but the addition of these client proteins shifts this towards a more closed conformation[394, 395]. The importance of GRP94 is demonstrated by the fact that homozygous mutations have never been described in humans. Homozygous knockout mutations of *GRP94* are incompatible with embryonic development[388], and the *grp94* gene is also highly conserved in species.

During times of extreme stress, the unfolded protein response (UPR) is a complex system that either restores homeostasis or identifies a cell for apoptosis. GRP94 expression is increased during endoplasmic reticulum stress[396] and plays an important role in this UPR. In addition to this, GRP94 also has a role in protein "quality control", where misfolded proteins are marked for cytosolic breakdown by the proteasome rather than secretion[380, 397].

The hypothesis that hypofunctioning mutations of *grp94* can affect IGF-I production and body size is supported by two observations. First, a mouse model with ablated striated muscle GRP94 has significantly reduced IGF-I concentrations and is 30% smaller than controls[381]. Second, *in vitro* studies of the cellular response to stress in the form of serum withdrawal demonstrate that *grp94 -/-* cells have an impaired response associated with reduced concentrations of IGF–II[387, 388].

In evaluating the clinical significance of GRP94 in IGF-I production, *grp94* gene sequencing was performed in 16 children with a combination of normal GH secretion, with short stature and low IGF-I concentrations[398]. One subject had a mutation of potential significance, and had a clinical phenotype suggestive of primary IGF-I deficiency. This included *in utero* growth restriction (birthweight z-score -2.82), postnatal growth failure, microcephaly and undetectable IGF-I at

4 months of age. He had a heterozygous substitution of C to T with a subsequent change in amino acid 300 for proline to lysine (P300L). His mother was also a carrier of this mutation and her height z-score was -0.9.

Functional studies of the effect of this mutation on GRP94 function are described in Appendix J, and demonstrated a reduction of IGF-II secretion to 58% when compared with wild type GRP94. The prevalence of this mutation in the normal population is up to 4%, which raised the question of whether this mutation contributes to variations in human IGF-I concentrations and height. The central hypothesis for this population-based study is that hypomorphic GRP94 heterozygous mutations are a cause of primary IGF-I deficiency and short stature.

3.2.2 AIM

The aim of my analysis was to determine if the P300L mutation is associated with short stature and low IGF-I concentrations in various population-based cohorts in whom P300L carrier status, height and/or IGF-I concentrations were available.

3.2.3 METHODS

Collaborations were formed between teams with the distinct cohort studies in which IGF-I concentrations were measured and height data were available. *Grp94* gene sequencing was performed where DNA was available, and subjects were characterised as carriers of the P300L mutation or wild type GRP94.

Population 1: Health In Men Study (HIMS, Australia)

The HIMS cohort study was initially designed to determine if screening for abdominal aortic aneurysms (AAAs) in at-risk adults reduced mortality and improved quality of life. 19,352 males over 65 years of age were identified from electoral databases and invited to attend baseline screening. Of these, 12,203 were recruited[399]. This study has demonstrated an insignificant reduction in

mortality from AAA through screening[400], shown an association between glucose control and aortic diameter[401], and identified predictors of cognitive function in men over 80 years of age[402]. This study provided an opportunity for collaboration as IGF-I concentrations were measured to evaluate a potential association between the GH/IGF-I axis and cardiovascular outcomes[399]. In addition, peripheral blood was collected for genetic analysis as part of this study[403].

Population 2: Longevity Genes Project (New York)

The aim of the longevity genes project is to identify genes that are associated with health and independence at 95 years of age, in a cohort of subjects who are of Ashkenazi Jewish descent[404]. This study recruited centenarians and their offspring. Blood samples were collected for analysis of cardiovascular risk factors[405] and other potential biomarkers of longevity[406]. This study has shown that low IGF-I concentrations are associated with longevity in females, and that low serum IGF-I is also associated with longer survival in subjects who had a prior diagnosis of cancer[407].

Population 3: Danish Cohort Study

This cohort includes 15,663 subjects in 5 separate population-based Danish studies[408]. This is the largest cohort from which data were available on P300L phenotype, but IGF-I concentrations were not measured in these subjects. However, height measurements were available.

Given the age-related decline in adult height, subjects were grouped according to age (20-39 years, 40-59 years, 60-79 years) and sex for the analysis of the effect of P300L carrier status on height and IGF-I concentrations.

Statistical Analysis

For normally distributed continuous variables, mean (SD) were compared between P300L carriers and controls using independent samples t-tests. Non-

normally distributed data were summarised as median (IQR) and compared using nonparametric (Mann Whitney U) tests. Extremely high IGF-I values in an elderly population may represent pathology (e.g. acromegaly) or laboratory errors (e.g. assay artifact from IGFBP interference). For this reason, all IGF-I measurements above +3SD from the mean (above 315.5 ng/ml) were excluded as biologically implausible from the HIMS Study (26 wild type, 2 P300L carriers). Data were analysed using SPSS version 22.0 (IBM, New York, USA).

3.2.4 RESULTS

Health in Men Study (Australia)

IGF-I concentrations, height and age were available in 3862 men over 65 years of age in the HIMS study. Of these, 112 were heterozygous for P300L mutation (carrier rate 2.9%), and one was homozygous for this mutation. IGF-I concentrations were lower in P300L carriers (p=0.037). There were no significant differences between groups in the available known confounders of IGF-I concentration: age (p=0.6); or body mass index (p=0.73) (Table 3.2.1). One subject was homozygous for the P300L mutation, and he had an IGF-I concentration of 226 ng/ml.

Table 3.2.1: IGF-I and IGFBP-3 concentrations, height, age and BMI according to P300L carrier status. *IGF-I concentrations were not normally distributed and are represented as median (IQR). Data shown are mean (SD) with Student's t-test used to compare groups.

	Wild Type GRP94 (n=3595)	**P300L (n=98)**	**p**
IGF-I (ng/ml)	140.3 (53.8)	130 (54.8)	0.037
IGFBP-3 (mg/L)	3.8 (0.9)	3.7 (1)	0.25
Height (cm)	171.9 (6.7)	173 (6.4)	0.13
BMI (kg/m^2)	26.6 (3.7)	26.7 (3.8)	0.73
Age	77.1 (3.6)	76.9 (3.5)	0.57

Longevity Genes Project (New York)

Of the 1369 genotyped subjects (642 male) in this cohort, there were 28 carriers (14 male) of P300L. Female carriers were slightly shorter than wild type, whereas there was no difference between males (Table 3.2.2). Only ten P300L heterozygotes and 22 wild type subjects had IGF-I concentrations measured. There was no difference in IGF-I concentrations when corrected for age.

Table 3.2.2: Height and IGF-I in P300L carriers and controls in the Longevity Genes project. Mean (SD) shown. ns=not significant

	P300L	**Wild Type**	**p**
Males			
Number	14	628	
Age, yrs	78.1 (13)	72.6 (8.5)	ns
Height, cm	170.7 (6.9)	174 (8.6)	ns
Females			
Number	14	713	
Age, yrs	82.3 (13.2)	71.5 (8.7)	ns
Height, cm	154.9 (4.6)	159.5 (7.4)	<0.01

Danish Cohort

P300L genotype was available in 15,633 subjects over 20 years of age (619 carriers). This represents a population carrier frequency of 4%. No significant growth attenuating effect of P300L carrier status was noted in any age- and sex-stratified subgroup of this population. In fact, female carriers of P300L aged between 20 and 59 years of age were taller than wild type counterparts (Table 3.2.3).

Table 3.2.3: Height of male and female subjects in the Danish cohort, according to P300L carrier status.

	Male					**Female**				
	P300L		**Wild-type**		**p**	**P300L**		**Wild-type**		**p**
	n	**Height**	**n**	**Height**		**n**	**Height**	**n**	**Height**	
20-39 years (n=1096)	40	181.4 (5.8)	1056	180.9 (6.4)	0.6	50	169.5 (5.4)	1360	167.4 (6.3)	0.01
40-59 years (n=4369)	157	178.8 (7.3)	4212	178.4 (6.8)	0.6	181	166.6 (6.8)	4100	165.6 (6.2)	0.04
60-79 years (n=2469)	102	174.6 (6.1)	2367	174.9 (6.4)	0.6	87	162.2 (5.3)	1846	162.1 (6)	0.9
>80 years (n=33)	0		33	174.8 (8.8)	n/a	2	162 (8.5)	41	159.3 (6.2)	0.7

3.2.5 DISCUSSION

Despite convincing *in vitro* data to support the deleterious effect of the P300L mutation of *grp94* on IGF processing, we failed to demonstrate the expected clinical phenotype of short stature and reduced IGF-I concentrations in heterozygous carriers of this mutation. In the single subject who was homozygous for this mutation, IGF-I concentration was within the normal range for age.

The absence of a clear clinical phenotype in the larger population studies makes it likely that the P300L mutation seen in the original clinical patient was an incidental finding in the initial subject with unexplained primary IGF-I deficiency, microcephaly and severe short stature. This is supported by the fact that his mother was also a carrier of this mutation, and she had a height z-score of -0.9.

Numerous potential explanations for this lack of clinical significance are proposed. Firstly, the *in vitro* experiments measured GRP94 activity in the absence of a coexisting allele with an unaffected *grp94* gene. Although one homozygous subject was included, almost all subjects were heterozygous for P300L and had a normal gene that may have been compensatory. It is also possible that feedback mechanisms such as increased GH production could overcome the effect of a hypomorphic variant of GRP94 and result in normal IGF-I production and growth. GH measurements were not available to investigate this further.

3.2.6 CHAPTER CONCLUSION

In this chapter, we have taken a candidate gene with biological and *in vitro* plausibility to the population level to determine if the genetic and biochemical data correlate with a clinical phenotype. Although the results were disappointing, it highlights the challenges in identifying prevalent genes that may have a mild

clinical phenotype. These data required significant contributions from numerous collaborators and ultimately resulted in negative data.

Through this process, I learned to analyse phenotypes across numerous populations and gained insight and hands-on experience in using cell-based assays to perform *in vitro* studies. I also learned to appreciate the complimentary roles of clinical phenotyping, querying genetic databases and lab-based research in evaluating the significance of novel genetic mutations.

CHAPTER 3.3: GENETIC APPROACH TO INSULIN-LIKE GROWTH FACTOR-I RESISTANCE

In the previous chapter, we started with a relatively common gene mutation of potential significance and studied the clinical phenotype in large populations. Here, we describe the reverse process of starting with a rare clinical phenotype and taking a genome-wide approach to identifying genetic aetiologies.

In this chapter, I will describe the development of a multi-site genomic approach to identifying novel mutations in three of the leading children's hospitals in the United States: Children's Hospital of Philadelphia (CHOP); Boston Children's Hospital (BCH); and Cincinnati Children's Hospital Medical Center (CCHMC). We used this process to identify children with rare mutations causing IGF-I resistance.

3.3.1 IGF-I RESISTANCE

IGF-I plays a critical role in regulating prenatal and postnatal growth, and infants with IGF-I deficiency have significant intrauterine growth restriction and postnatal growth failure. In addition, sensorineural hearing loss[409], microcephaly and developmental delay are part of this clinical phenotype[153].

Haploinsufficiency of the IGF-1R can have variable effects on IGF-IR expression and reduced IGF-I signaling (3.1.1)[410]. High IGF-I concentrations and short stature are consistent components of the phenotype. At the more severe end of the clinical spectrum with large deletions involving the entire *IGF1R* gene, cardiac defects, pulmonary hypoplasia and congenital diaphragmatic hernia may be seen[411, 412]. It is postulated that these additional clinical features may be due to the effects of deletions on genes flanking *IGF-1R*[410-412].

Recently, a novel mechanism for IGF-I resistance has been reported as a cause of short stature. Mutations in pregnancy-associated plasma protein A2 (PAPP-A2)

have been shown to impair proteolytic cleavage of IGF-I from the ternary complex and lead to high concentrations of circulating total IGF-I but reduced free IGF-I activity[413, 414]. The clinical phenotype was subtler with height and head circumference z-scores just below normal, with high circulating IGF-I and –II concentrations.

3.3.2 AIM

The aim of this study was to utilise electronic health records (EHRs) to identify children with a clinical phenotype consistent with IGF-I resistance and to identify novel genetic mechanisms for this phenotype.

3.3.3 METHODS

Genomics Research and Innovation Network (GRIN)

The GRIN is a collaborative network between CHOP, BCH and CCHMC. This was established in 2015 and provides an infrastructure for sharing expertise, protocols, ethics committee approval of parallel protocols, EHR search criteria, genetic material and research data. The identification of novel genetic mechanisms of the rare phenotype of IGF-I resistance was adopted as one of the three pilot studies as this infrastructure was being developed. The CHOP Institutional Review Board provided ethical approval for this study. A collaborative research agreement was also signed to facilitate the sharing of research data and protocols between institutions.

Subject identification – Height criteria

The EHR was searched to identify all children attending CHOP who had an age- and sex-specific height z-score of less than -2 using the Center for Disease Control growth standards[415].

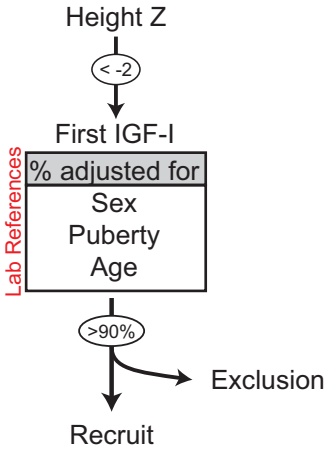

Figure 3.3.1: The stepwise approach to using the electronic health record identify children with high IGF-I concentrations and short stature

IGF-I concentration: Lab-Specific Factors

In the United States, the laboratory used for patient tests is often dictated by contracts negotiated between health insurance companies and individual laboratories. In addition, the IGF-I assay used by each laboratory changes over time and this affects the reported reference ranges for each reported measurement. We contacted the three most frequently used laboratories and requested information on the reference ranges for each of their IGF-I assays used between 2000 and 2016. One of the laboratories (QUEST) was unable to provide us with this information, and the information provided by another laboratory (LabCorp) was inaccurate on manual review.

As an alternative approach, representative samples were identified for each of the IGF-I assays in each laboratory using the electronic health record (EHR). The reference ranges listed in each assay were recorded manually for the pubertal and/or age ranges. Mean and standard deviations were generated from these, and an algorithm was created to query the EHR and generate percentiles for each test according to pubertal stage and/or age. Some assays reported z-scores directly and, where possible, these were extracted and converted to percentiles.

In order to validate this approach, we counted the number of samples with IGF-I concentrations above the 90th percentile for each assay to ensure that no assay was over represented on initial screen.

IGF-I concentration: Patient-specific factors

IGF-I concentrations increase during puberty, and precocious puberty in a child with short stature would erroneously be identified as IGF-I resistance if age-specific reference values were used. Pubertal status is not a discrete field in the EHR, but is generally included in the text of clinical notes. An example of patient data that needed to be reconciled manually is shown in Figure 3.3.2.

Medical Notes:

Examination: Pubic hair is is Tanner stage 3. Testes are 15 ml bilaterally.

IGF-I Results

IGF-1 (BL)	517
Comment:	
Reference Range:	
Tanner Range Mean	
16y 2 and 3 201 - 648 386	
16y 4 and 5 180 - 501 316	
IGF-1 Z-Score For Tanner 1	N/A
IGF-1 Z-Score For Tanner 2	1.0
IGF-1 Z-Score For Tanner 3	1.0
IGF-1 Z-Score For Tanner 4 / 5	2.2
Comment:	
Z-Scores calculated on asymmetric curves with Transformed data.	

Figure 3.3.2: Example medical note documentation of pubertal status and laboratory IGF-I report of IGF-I concentration requiring reconcilliation

As manually reviewing each chart to extract pubertal status was not feasible, a natural language query to extract pubertal status at the time of IGF-I measurement was developed. The following text was identified from medical records within 3 months of IGF-I measurement and extracted from the EHR for manual review: 100 characters before and 100 characters after the word if used): “Tanner”, “testes”, “testicular”, “testis”, “Pubic hair”, “breast”, as well as date of encounter. If this was in a string of text including a number of these words, all intervening text was extracted. Extracted data were then manually reviewed to modify IGF-I concentrations for pubertal status where necessary.

Exclusion Criteria

GH treatment can artificially increase IGF-I concentrations and was an exclusion criterion for this study. In order to exclude all forms of GH used, we excluded subjects receiving any of the following medications: Genotropin, Gentropin, Humatrope, Norditropin, Nutropin, Omnitrope, Saizen, Somatropin and growth hormone.

Further exclusion criteria were applied following manual chart review, to focus recruitment on children without co-existing disease or treatment that may independently affect IGF-I concentrations and/or growth. These included: chronic kidney disease; malignancy receiving treatment at the time of IGF-I measurement; high dose steroid treatment; known genetic diagnosis associated with short stature. In addition, children receiving GH treatment at the time of IGF-I measurement were excluded, unless IGF-I concentrations prior to treatment were also elevated. Children who attained height above -2 z-scores without treatment, or who had normal IGF-I concentrations at any time on chart review were also excluded.

Genetic Testing

In this collaborative study, Dr Andrew Dauber at CCHMC performed genetic testing and analysis. The process is summarised here for additional background, but performing this genetic testing was not my personal work.

Candidate gene testing comprised Sanger sequencing of the IGF-I receptor (*IGF1R*), and *PAPPA2*. If this was negative, whole exome sequencing was performed at the CCHMC Sequencing Core. This involved 1 ug of dsDNA undergoing 9 cycles of PCR amplification using the Clonetech kit (Clonetech Laboratories, CA, USA). This was sequenced using the Illumina HiSeq 2500 (Illumina Inc., CA, USA), and was analysed using the Broad Institute's Genome Analysis Toolkit (Broad Institute, MA, USA).

Variants of minor allele frequency were identified using public databases and excluded from analysis. In order to identify novel or *de novo* variants, dominant variants were eliminated if present in the public databases (e.g. Exome Aggregation Consortium website) and recessive variants were eliminated if a minor allele frequency of greater than 0.001 were noted. This reflects a disorder present in less than one in a million people. For potentially significant variants, parent samples were sequenced.

Following the identification of a candidate gene, additional searches were performed using OMIM, DECIPHER and PubMed to determine if this has been previously associated with human disease. *In silico* prediction tools, known gene function, animal model data and gene expression patterns were also reviewed to prioritise variants for further mechanistic studies.

3.3.4 RESULTS

This study was approved by the IRB at CHOP and parallel protocols were approved separately by the IRBs at CCHMC and BCH. For the purpose of this thesis, I will describe the results from the CHOP protocol.

We identified 68 children with IGF-I concentrations greater than the 90th percentile and height z-scores below -2. Of these, 54 children were excluded (Table 3.3.1), leaving 14 eligible for recruitment. Of the remaining 14 subjects, two no longer lived in the US, eight did not wish to participate, and four were recruited. Their clinical details are shown in Table 3.1.2.

Table 3.1.1: Excluded children from analysis
* rhabdomyosarcoma, hemophagocytic lymphohistiocytosis, acute lymphoblastic leukemia.
** trisomy 21, Turner syndrome, Angelman syndrome, neurofibromatosis type 1, retinitis pigmentosa, Aicardi Goutier syndrome, Noonan syndrome

Number	Reason for Exclusion
4	Chronic kidney disease
3	Malignant disease receiving treatment*
8	IGF-I concentration normal when corrected for puberty
15	Other IGF-I measurements in EHR were within normal range
10	Normal height without treatment
5	High dose steroids
7	Known genetic disorder**
2	Receiving growth hormone treatment

Table 3.1.2: Recruited subjects from CHOP

Age (yrs)	IGF-I z-score	Height z-score	Birth weight z-score	Relevant Clinical Details
7.5	3	-2.8	-1.8	Attention deficit and hyperactivity disorder, constipation, familial short stature.
8.6	1.9	-3.3	3	Hypotonia, speech and fine motor developmental delay, upslanting palpebral fissures.
7.8	1.6	-2.4	1.1	Sensorineural hearing loss.
3.4	1.7	-4.4	-2.8	Ventricular septal defect, imperforate anus

Exome sequencing was performed on all four subjects. No candidate genes were identified in Subjects 2, 3 or 4. Subject 1 had a novel missense variant in the IGF1R gene (Val1013Phe). His mother is not a carrier of this mutation, and her height is 5 feet 1 inch. His father was not involved in the study and his genetic sample was not available for review. This mutation is being regenerated in Andrew Dauber's lab, and functionality is being assessed.

3.3.5 DISCUSSION

In this study, we have developed an EHR approach to identifying children with a clinical phenotype of IGF-I resistance. We have applied this approach to patients attending CHOP's healthcare system and identified 14 subjects who may have this rare clinical phenotype. Thus far, we have identified one potentially novel mechanism for IGF-I resistance and this is being investigated further.

Although we have only identified one potentially pathogenic mutation, this study has been successful on a number of fronts. We have formed a collaboration amongst the growth centers in three of the largest children's hospitals in the US, and established three parallel research protocols to recruit children with abnormal growth. We have also developed a mechanism for data and protocol sharing between these institutions. CCHMC and BCH are still recruiting subjects and it is possible that these centers will identify additional mutations of interest.

At CHOP, we have overcome a significant barrier to growth research using the EHR. We have developed an algorithm to extract IGF-I z-scores and pubertal status from the entire population of children attending this center. We now have the capacity to generate IGF-I z-scores from the entire population of children who attend CHOP, regardless of laboratory or assay used. We have also developed a mechanism for reliably extracting pubertal status from the EHR. We plan to utilise these algorithms for future genetic and clinical studies involving our patient population.

3.3.6 CHAPTER CONCLUSION

I have participated in large collaborative studies aiming to identify and describe novel genetic causes of short stature in childhood. In Chapter 3.2, we started with a mutation that was potentially pathogenic and worked through functional studies to demonstrate a potential effect on IGF production. In Chapter 3.3, we started with a rare clinical phenotype and developed algorithms to identify potential subjects using the EHR.

These studies highlight the challenge of finding novel genetic aetiologies of short stature. Short stature is a multifactorial trait, of which genetics may be one factor. In evaluating the child with short stature, an underlying genetic mechanism is possible and many of these have been described[358]. However, as we have shown, finding and proving new genetic aetiologies can be difficult.

SECTION 4

NUTRITION AND THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS

CHAPTER 4.1: INTRODUCTION

GH is a key regulator of IGF-I production. IGF-I measurement is amongst the first screening tests performed in children with suspected GHD, and Section 2 of this thesis focused on measuring IGF-I concentrations using a new assay. In Section 3, I explored genetic aetiologies of disordered IGF-I production and action.

In this Section, I will focus on nutrition, another clinical aspect of childhood growth linked to the GH / IGF axis. This appears to be particularly important in the fetus and young infant, where nutritional status is most closely associated with IGF-I concentrations.

In children identified as having short stature or poor growth of unclear aetiology, nutritional evaluation is a key component of the diagnostic evaluation. In early infancy, weight is generally considered to be a marker of nutritional status but weight measurement does not differentiate fat mass (FM) from fat free mass (FFM). In this section, I will explore body composition measures in infancy with an emphasis on generating reference data and determining the optimal measures of size-adjusted body composition.

Having generated reference data for body composition in infancy, I will then consider whether IGF-I and –II measurement at birth is associated with body composition and rate of change in body composition in the first two months of life. Thus, this section of the thesis will provide a new approach to describing nutritional status in infancy and will explore the relationship between these measures and the GH/IGF axis.

CHAPTER 4.2: NUTRITION, GROWTH AND THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS

Publication

Hawkes CP, Grimberg A. Insulin-like growth factor-I is a marker for the nutritional state. Pediatr Endocrinol Rev. 2015;13(2):465-77. (Appendix K)

4.2.1 INTRODUCTION

Faltering linear growth in childhood may be a sensitive but nonspecific sign of disease.[42] Two of the diagnostic categories that can present with faltering growth are insufficient nutrition and disorders of the GH / IGF-I axis. GH stimulates IGF-I production, and measurement of IGF-I concentration is commonly used to screen for disorders of the axis (1.2.4.1)[2, 9]. Nutritional status and GH signaling can both affect IGF-I concentrations, and an understanding of these overlapping effects on IGF-I production is essential in order to interpret IGF-I measurements in children.

4.2.2 AIM

The aim of this review is to provide an overview of the effects of nutrition on the GH/IGF-I axis and discuss the clinical implications of these interactions throughout childhood, both in undernutrition and overnutrition.

4.2.3 THE GROWTH HORMONE / INSULIN-LIKE GROWTH FACTOR-I AXIS AND POTENTIAL INTERACTION WITH NUTRITION

GH is secreted by the somatotrophic cells of the anterior pituitary gland in a pulsatile fashion, with approximately five to eight peaks each day[416]. This pulsatile secretion is stimulated by hypothalamic GHRH, with endocrine feedback mechanisms further regulating this system. These include somatostatin, IGF-I, and ghrelin (Figure 4.2.1). Nutritional status can interact with the

GH/IGF-I axis at numerous points in the pathway, at both the hormone secretion and post-receptor signaling levels.

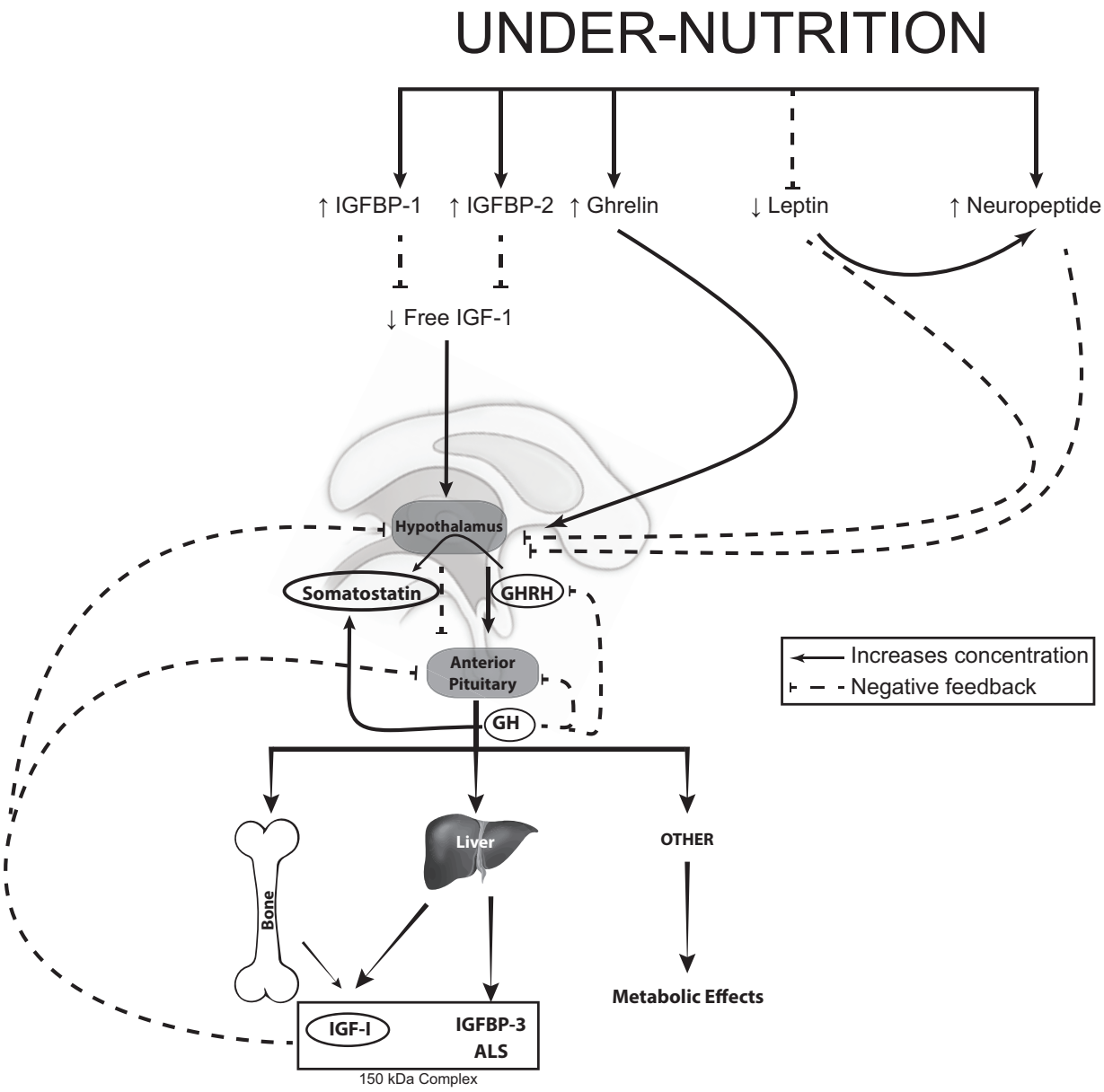

Figure 4.2.1: The effect of undernutrition on GH secretion. Leptin modifies hypothalamic regulation of GH secretion through hypothalamic receptors, and in severe undernutrition insufficient leptin reduces GH production both directly and through the consequent increase in Neuropeptide Y. Anterior pituitary GH secretion is controlled by hypothalamic GHRH and somatostatin, as well as feedback mechanisms through IGF-I, ghrelin and GH concentrations.
GH= Growth Hormone, IGF-I = Insulin-like Growth Factor-I, IGFBP = Insulin-like Binding Protein, ALS = Acid Labile Subunit, GHRH = Growth Hormone Releasing Hormone

4.2.3.1 The impact of undernutrition on the regulation of growth hormone secretion

In chronic undernutrition, alterations in leptin and neuropeptide Y (NPY) concentrations can reduce GH secretion. Leptin is produced by adipose tissue[417], and normal concentrations of circulating leptin are required for GH secretion. In animal studies where leptin anti-serum was used to reduce serum leptin concentrations, there was an associated reduction in GH secretion[418]. The mechanism for this interaction may be through a direct effect on hypothalamic leptin receptors, or indirectly through NPY. Leptin suppresses hypothalamic NPY production, and NPY suppresses GH release[419, 420]. Thus, in starvation there are reduced leptin concentrations and increased hypothalamic production of NPY, which may reduce pituitary GH secretion.

The oxyntic glands of the gastric fundus secrete Ghrelin and this binds to the GH secretagogue receptor 1a, stimulating GH secretion by the anterior pituitary. Three days of caloric restriction prior to GH stimulation testing may increase the peak stimulated GH concentration achieved during testing of GH-sufficient children[203]. Ghrelin concentrations are increased during fasting and suppressed with feeding[421], and may mediate this effect. However, ghrelin administration in chronic malnutrition is not associated with a rise in GH concentration[422].

4.2.3.2 The impact of undernutrition on growth hormone signaling

The GH receptor is a type 1 cytokine receptor that is expressed predominantly by hepatocytes. GH binding results in rotation of one of the monomers of this dimeric receptor, and the intracellular domain binds JAK2[359]. This activates numerous signaling pathways, specifically the STAT -1, -3, -5a, -5b, MAPK and PI3K pathways[376] (Figure 4.2.2). Activation of these intracellular signaling pathways, primarily STAT5b, stimulates transcription of IGF-I (This pathway is described in more detail in 3.1.1).

Undernutrition may affect GH signaling at multiple points on the pathway and cause a state of GH resistance. In animal and cell-based models, caloric

restriction is associated with a reduction in GH receptor mRNA transcription[423, 424]. Insulin increases hepatic GH receptor availability[425], and reduced insulin concentration during fasting may play a role in the reduction of GH receptor transcription. Calorie and protein malnutrition can also cause GH resistance through effects on post-receptor signaling.

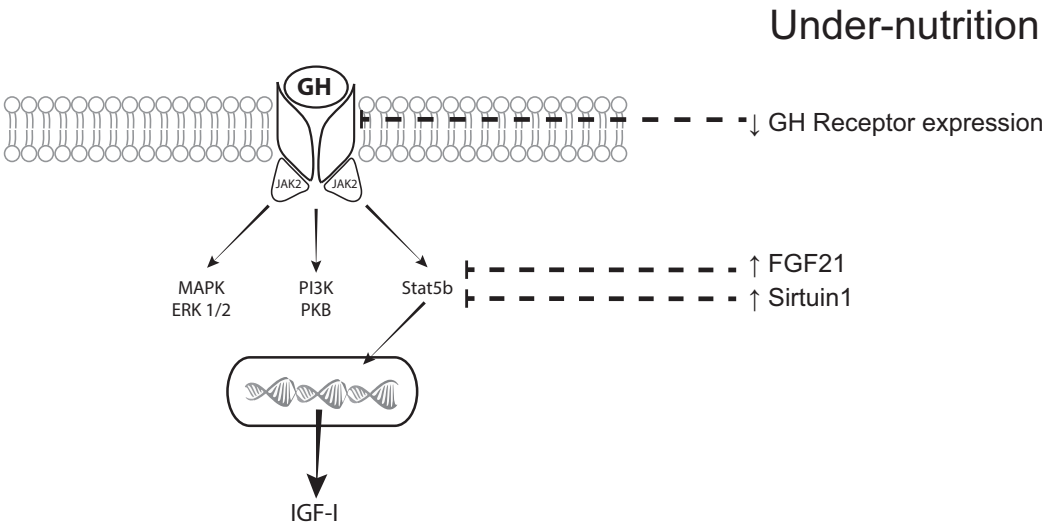

Figure 4.2.2: Nutrition and Intracellular Growth Hormone Signaling.
Growth hormone binds to the extracellular domain of the dimeric GH receptor, and results in activation of multiple intracellular signaling pathways. GH receptor expression is reduced in caloric restriction. FGF21 and Sirtuin-1 concentrations are increased in fasting, and both of these reduce tyrosine kinase phosphorylation of STAT5b.
JAK2 = Janus kinase 2, MAPK = Mitogen-activated protein kinase, ERK = Extracellular-signal regulated kinase, PI3K = Phosphoinositide 3 kinase, PKB = Protein kinase B (also known as AKT), Stat = Signal transducer and activator of transcription, FGF21 = Fibroblast growth factor 21, GH = growth hormone, IGF-I = insulin-like growth factor-I

Fibroblast growth factor 21 (FGF21) is produced by adipocytes and hepatocytes, and concentrations are increased in fasting[426]. FGF21 reduces STAT5b phosphorylation and increases Suppressor of Cytokine Signaling 2 (SOCS2) expression, both of which decrease IGF-I production[427]. FGF21 also increases IGFBP-1 expression, which further reduces IGF-I bioavailability for signaling[428]. Another potential mechanism involves Sirtuin-1, a deacetylase that mediates the metabolic response to fasting through its effects on glucose and lipid metabolism[429]. Sirtuin-1 also inhibits the tyrosine phosphorylation of STAT5[430], and represents an additional cellular mechanism of GH resistance in malnutrition. Zinc[431], magnesium[432] and vitamin B6[433] deficiencies may also be

associated with GH resistance and reduced IGF-I, although the mechanisms of each of these are unknown.

4.2.4 Nutrition and Insulin-like Growth Factor-I from the Fetus through Adolescence

Prenatal GHD has minimal effect on birth size[219], whereas children with IGF-I receptor mutations or primary IGF-I deficiency are born with severe intrauterine growth restriction[153]. The GH-independent regulation of fetal IGF-I is poorly understood, but the pattern of changes in fetal IGF-I concentration mirrors weight gain. Fetal serum IGF-I concentrations increase steadily throughout the third trimester[350], which coincides with the period of most rapid increase in fetal weight. In addition, umbilical cord IGF-I concentrations at birth correlate with birth weight[275] (Chapter 2.4).

Following birth, IGF-I levels are closely associated with weight and nutritional intake[222, 434]. While adequate caloric intake is necessary for symmetrical infant growth, caloric excess will increase infant weight more than length and head circumference[435]. Changes in circulating IGF-I concentrations can reflect increases in protein and caloric intake, and IGF-I measurement has been suggested as a method of monitoring feeding, particularly in preterm infants[436]. In early infancy, formula fed infants have increased adiposity and higher IGF-I concentrations than breastfed infants[313, 437, 438]. The effect of milk in increasing IGF-I concentrations has been studied further in 2 to 3 year-olds. In this population, circulating IGF-I is increased by 30% when milk intake increases from 200 to 600 ml/day, an effect predominantly due to the casein component of milk[439-441].

In prepubertal children, lower BMI is associated with lower serum IGF-I concentrations[442]. Although reduced caloric intake is associated with low circulating IGF-I, nutritional factors beyond calories and protein should also be considered in children with unexpectedly low IGF-I levels. Zinc deficiency, for

example, in peripubertal children is associated with lower IGF-I and IGFBP-3 concentrations. IGF-I concentrations improve with zinc replacement in these children[431, 443]. Iodine deficiency is also associated with low circulating IGF-I, but replacement of iodine deficiency can have the effect of reducing IGF-I concentrations further[444]. This finding is most likely related to the effect of iodine replacement on thyroid function. Excess iodine can suppress thyroid function through the Wolff-Chaikoff effect. IGF-I concentrations are also reduced in hypothyroidism[445, 446].

IGF-I concentrations increase during puberty, and energy requirements are also increased during this time[447]. The sex steroids of puberty increase pituitary GH secretion, which in turn increases IGF-I production. The combination of increased sex steroids, GH and IGF-I cause the pubertal growth spurt. Insufficient nutrition can delay the onset of puberty[448], and leptin concentrations above a certain threshold appear to be required for puberty to proceed[449, 450]. Low IGF-I concentrations in adolescents suffering from malnutrition can appear even lower relative to reference ranges if age-based rather than pubertal stage-specific reference ranges for IGF-I are reported.

4.2.5 UNDERNUTRITION AND INSULIN-LIKE GROWTH FACTOR-I

4.2.5.1 Populations at risk for GH/IGF axis effects from insufficient intake

Sufficient nutrition is a balance between caloric intake and energy expenditure. Caloric intake may be affected by food availability and appetite, and increased energy expenditure may put active or pubescent children at risk for undernutrition. In this section, we will review populations who are at particular risk.

Food availability can be a significant issue for many families, and may not be immediately apparent during a clinical visit. The worldwide prevalence of undernourishment is estimated at 11.3%[451], and an estimated 50 million people in the United States are uncertain of having enough food[452]. This may affect the

type and quantity of food available[453, 454]. As will be discussed later in this section, even a transient interruption to calorie or protein availability can result in a reduction in IGF-I concentrations (Figure 4.2.3)[455]. This may be relevant in families where the next paycheck is required before food can be purchased.

Dietary intake may be decreased despite adequate food availability due to certain feeding behaviors and/or diminished appetite. Sometimes the dietary intake is inadvertently over-restricted by the parent(s) and/or child due to fear of obesity[456-458] or hypercholesterolemia[458], to the extent that nutritional growth stunting ensues. Unstructured mealtimes, particularly those with distractions, as well as food aversions and dysfunctional parent-child interactions related to eating can all lead to failure to thrive, a topic extensively reviewed elsewhere[459]. Undernutrition can also result from decreased appetite, which may be endogenous (such as from delayed gastric emptying) or secondary to medications. Attention deficit and hyperactivity disorder (ADHD) affects approximately 7% of children[460], and methylphenidate or dexamphetamine are commonly used to treat this disorder. These medications are associated with appetite suppression and subsequent weight loss[461]. In a small study that included healthy children treated with methylphenidate, reduced weight and BMI were seen within 4 months of treatment and an associated reduction in IGF-I concentration was observed[462].

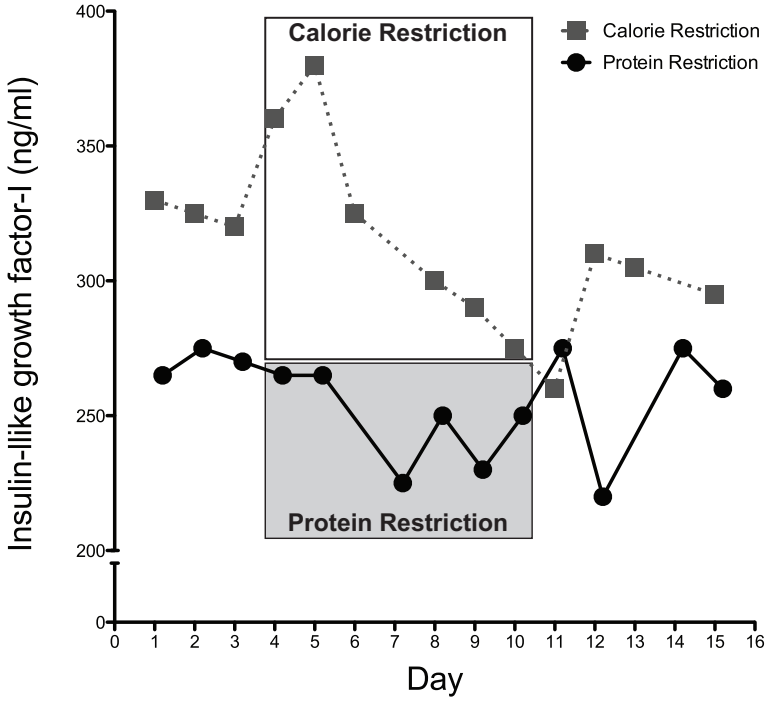

Figure 4.2.3: Seven days of 50% caloric restriction (35 Cal/kg) resulted in a reduction in mean IGF-I amongst eight prepubertal children. Mean IGF-I also decreased during 33% protein restriction (0.66 g/kg) in 6 other prepubertal children. Figure modified from Smith *et al*[455]

4.2.5.2 Populations at risk for GH / IGF axis effects from increased demands

The recommended caloric intake in children and adolescents is dependent upon energy expenditure. The estimated energy requirements in childhood varies according to age and sex, and ranges from 520 kcal/day in the infant female to 3,152 kcal/day in the adolescent male. This is increased further in the setting of regular rigorous exercise[447]. Nutritional intake should be considered in the context of an individual's activity level, as similar dietary intakes may be sufficient for a sedentary child but insufficient for an extremely active child. This chronic nutritional insufficiency may affect IGF-I concentrations.

Failure to meet the nutritional demands of competitive sport is prevalent amongst adolescents[463-465]. For example, a study of adolescent soccer players showed that the mean caloric intake was almost 500 kcal/day less than estimated requirements[466]. At the extreme end of the spectrum of undernutrition in sport, a triad that includes low energy availability, menstrual dysfunction and reduced bone mineral density has been well described in females[467]. Up to 8% of athletic

females have two or more features of this triad[468], and low estrogen and IGF-I levels are thought to play a role in the pathogenesis of the low bone mineral density[469]. However, the adverse effects of undernutrition in males should not be underestimated, and the International Olympic Committee's recent guidelines has replaced the term "Female Athlete Triad" to "Relative Energy Deficiency in Sport"[470]. This expands the definition to include both male athletes and other health repercussions beyond the original three, and acknowledges the variable degrees of severity of this condition[470]. IGF-I concentrations in gymnasts are lower than in controls[471], and this may be a associated with the high prevalence of insufficient nutritional intake in this sport[463].

Reduced IGF-I concentrations are also seen in systemic diseases associated with increased energy requirements and/or malabsorption. These conditions include inflammatory bowel disease, cystic fibrosis, cardiac disease (particularly cyanotic conditions and congestive failure) and acquired immune deficiency syndrome (AIDS). Ensuring adequate nutrition in each of these conditions is one of the key components of management. Poor linear growth is seen in children with severe disease, and low IGF-I concentrations may reflect insufficient nutritional intake in these children.

4.2.5.3 Populations at risk for GH / IGF axis effects from malabsorption

Low IGF-I concentrations may be an early sign of malabsorptive disorders, even in the absence of gastrointestinal symptoms. Celiac disease may present with a broad spectrum of symptoms and signs ranging from asymptomatic mild malabsorption (called monosymptomatic celiac disease, where the only symptom is growth failure[472]) to severe malnutrition and secondary failure to thrive. Even in minimally symptomatic children, IGF-I concentration is lower than controls at diagnosis and normalises with a gluten-free diet in parallel to increasing BMI[473]. In children with established celiac disease, gluten exposure leads to a reduction in circulating IGF-I concentration proportional to the degree of small bowel mucosal inflammation[474]. The correlation of IGF-I levels with celiac disease activity has been demonstrated in many pediatric[474-476] and adult[477] studies, and

IGF-I has even been suggested as an additional marker for monitoring celiac disease activity for this reason[475].

Complicating the observation of low IGF-I concentrations in children with undiagnosed celiac disease is the putative link between celiac disease and GH deficiency. Ferrante *et al* performed GH stimulation testing in adults with new onset celiac disease, and found that a quarter of patients were characterised as having impaired GH secretion on these tests[478]. It should be noted, however, that the stimulation tests were performed at baseline and not repeated following initiation of a gluten-free diet. As mentioned previously, blunted response to GH stimulation testing was found in children with protein malnutrition[479]. This responded to dietary replacement, and should caution physicians against performing stimulation testing prior to dietary management. However, a potential diagnosis of comorbid GHD should be considered in children with celiac disease who don't have improved linear growth despite adherence with a gluten-free diet[480, 481].

Poor growth and low IGF-I concentrations may also be a presentation of pediatric Crohn's disease. One tenth of affected children are more than two standard deviations below the mean for height at diagnosis[482], and they have numerous reasons for having reduced IGF-I concentrations. These include malnutrition complicated by increased metabolic rate during illness, increased inflammatory cytokines, and delayed puberty[473]. IGF-I concentrations increase during disease remission[483].

4.2.5.4 The effect of mild protein or caloric restriction on the GH/IGF-I axis

The GH/IGF axis is sensitive to less severe and transient nutritional restrictions. Smith *et al*[455] measured serum IGF-I levels in prepubertal children prior to and after six days of 50% calorie or 33% protein restriction. IGF-I levels were reduced during the periods of decreased intake of either calories or protein (Figure 4.2.3). This reduced IGF-I concentration during restriction is due to GH resistance rather than decreased GH secretion, as has been shown in similar

experiments in children with confirmed GHD receiving unchanged GH doses during dietary adjustments. In these children, IGF-I production in response to GH treatment was reduced during fasting[484]. In normal, fasted children, IGF-I levels returned to baseline concentrations following resumption of a normal diet[455]. Similar effects on IGF-I concentrations have also been shown in adults undergoing a short period of fasting[485]. In addition to reduced IGF-I concentrations, caloric restriction also renders the growth plate less responsive to IGF-I and GH through reduced expression of growth plate GH and IGF-I receptors[486].

4.2.5.5 The effect of severe protein or caloric restriction on the GH/IGF-I axis

The GH/IGF-I axis has been studied in children with extreme deficiency of protein (kwashiorkor), calories (marasmus) or both protein and calories (marasmic kwashiorkor)[487]. In all three of these types of undernutrition, low IGF-I and increased GH concentrations are seen. When GH stimulation testing was performed in children with protein malnutrition prior to nutrition therapy, the baseline GH concentration was higher and the increase in GH secretion upon stimulation testing was lower than in controls or following refeeding[479].

Hintz *et al*[488] studied 27 Thai children under 5 years of age, and Soliman *et al*[479] studied 51 Egyptian children under 3 years of age with kwashiorkor, marasmus or marasmic kwashiorkor and both showed similar results. Prior to nutritional repletion, GH concentrations were increased and IGF-I concentrations were decreased relative to controls. There were no significant differences in the magnitude of these changes between the three groups of severe undernutrition. Serum IGF-I and albumin levels did not correlate at baseline[488], suggesting that reduced IGF-I concentration is not merely a result of decreased amino acid availability or reduced hepatic protein synthesis.

Nutritional replacement in children with severe protein and/or calorie malnutrition can normalise the GH/IGF-I axis. Within two weeks of re-feeding,

IGF-I concentrations can double in severely malnourished children, to a level within 2 standard deviations of the mean for the population[489]. After 50 days of intensive inpatient nutritional therapy[488], basal GH and IGF-I levels are indistinguishable from those of controls (Figure 4.2.4). Interestingly, IGF-I z-scores are not associated with weight-for-height z-scores in severe malnutrition, but they have a linear relationship after two weeks of refeeding[489]. Similarly, nutritional interventions are associated with early increases in IGF-I levels, even before changes in anthropometric measures are observed.[490]

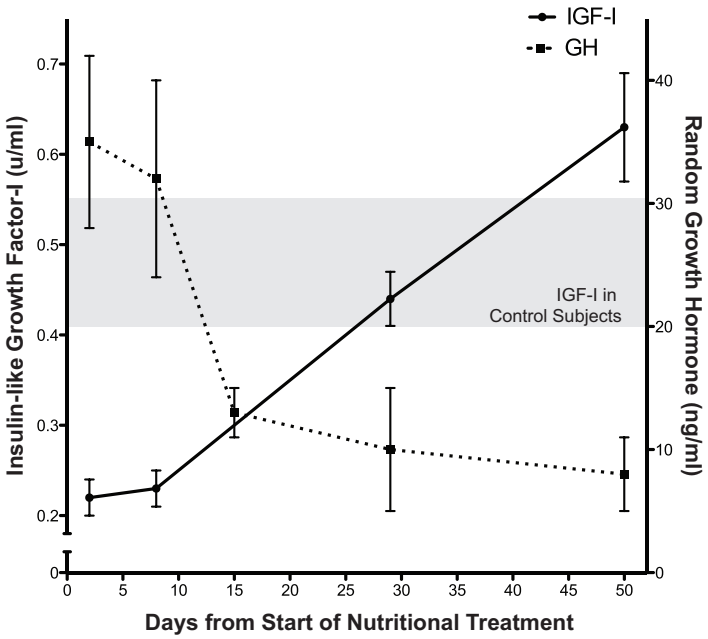

Figure 4.2.4: The effect of nutritional treatment on serum IGF-I and GH in a group of children with severe protein-energy malnutrition.
Mean ± SEM IGF-I concentrations in healthy control subjects are shown in grey. Figure modified from Hintz et al.[488]

4.2.6 EXCESS NUTRITION AND INSULIN-LIKE GROWTH FACTOR-I

Approximately one fifth of children in developed countries are overweight and, even in developing countries with a high prevalence of undernutrition, the percentage of children who are overweight is increasing[491, 492]. Obese children are taller than non-obese in childhood, but enter puberty earlier[493] and have comparable adult heights[494-496]. Children who develop obesity earlier in

childhood are more likely to have taller stature in childhood than their peers[497]. As will be outlined in this section, obesity affects the GH/IGF-I axis, and this may play a role in this accelerated statural growth.

4.2.6.1 Obesity and the GH/IGF-I Axis

Obesity is associated with reduced spontaneous total GH secretion through a reduction in both pulse frequency and amplitude[498, 499]. Furthermore, the circulating half-life of GH in obese subjects is reduced to 11 minutes in comparison to 15 minutes in non-obese controls[498]. Decreased amplitude of GH secretion is seen also during provocative testing, where peak stimulated GH concentrations in obese children[500-502] and adults[503] are lower than in non-obese controls. This can lead to children with increased BMI being misclassified as having GHD, even when BMI is within the normal range[502].

Despite reduced circulating GH levels, IGF-I concentrations in obese individuals are similar to, or higher than, non-obese controls (discussed later in this section)[500, 504-506]. Growth hormone binding protein (GHBP) is the extracellular component of the GH receptor, and serum concentrations of GHBP are increased in obesity. This may reflect increased GH sensitivity at the receptor level in obese children.[507] Increased GH sensitivity in children with higher BMI is supported by a study of IGF-I concentrations in children following administration of a fixed dose of GH. Children with high normal BMI had a greater rise in serum IGF-I concentrations when compared with those with low normal BMI[508].

Numerous studies have shown an association between IGF-I and leptin concentrations[509-511]. Animal studies show that administration of low doses of exogenous leptin result in a transient change in GH secretion but sustained reduction in circulating IGF-I concentrations. At higher doses, there is suppressed appetite and increased IGF-I levels[512]. This effect of leptin on IGF-I production appears to be independent of GH. However, GH can play a role in regulating leptin, as high doses of GH can increase leptin mRNA expression[513].

Leptin may also play an independent role in linear growth, through its direct action on the growth plate. At the growth plate[514], leptin synergises with thyroid hormone in regulating chondrocyte differentiation.[515] A phenomenon of "growth without GH" has been described in children where obesity and leptin may play a role in transiently maintaining normal growth velocity in children with GHD. This can be seen in children with coexisting hypothalamic obesity and GHD associated with craniopharyngioma[516], or in other children with severe obesity and GHD[517]. GHD may be masked by normal growth rate in these children, and they should be followed closely as this normal rate of growth may not be maintained and intervention with GH therapy may be indicated.

4.2.7 INSULIN-LIKE GROWTH FACTOR-I CONCENTRATIONS AND BIOAVAILABILITY IN OBESITY

Undernutrition consistently results in a reduction of IGF-I concentrations, but the opposite effect is not seen in obesity. Reinehr *et al* measured serum IGF-I concentrations in 319 obese children prior to, and after, a weight loss intervention program. They found no difference between baseline IGF-I concentrations in obese subjects or controls, and no association between weight loss and reduction in IGF-I levels[518]. This lack of association between obesity and increased IGF-I concentrations has been replicated elsewhere[504, 519]. In a population-based study of over six thousand adults, an inverse U-shaped association between IGF-I concentration and BMI was shown, with peak IGF-I concentrations at a BMI range of 22.5 to 25 kg/m^2 in males and 27.5 to 30 kg/m^2 in females[506].

Although total IGF-I concentrations may not be significantly increased in obesity, the amount of free IGF-I relative to total IGF-I is increased[504]. This increase in IGF-I bioavailability is largely due to the effect of nutrition on the IGF binding proteins (IGFBPs). Increased BMI and high insulin levels are associated with a reduction in levels of IGFBP-1[520, 521] and IGFBP-2[504], which

can increase free IGF-I concentrations. IGFBP-3 concentrations are similar in obese and normal weight children[504].

4.2.7.1 Potential implications of GH/IGF-I changes from overnutrition

The association between obesity and risk for several diseases of adulthood is mediated, at least in part, by the increase in GH sensitivity and IGF-I bioavailability from overnutrition. Obesity is a risk factor for cancer[522], and increasing BMI by 5 kg/m^2 will increase the relative risk of developing many different types of malignancies[523]. Overweight patients who develop cancer have a worse prognosis than those with normal weight[522, 524]. The mechanism for this association is not fully understood, but thought to be multifactorial and include the IGF-I signaling pathway[525]. There is evidence at the extreme ends of the spectrum of IGF-I to support this link: the incidence of colon cancer is increased in acromegaly[526]; and congenital IGF-I deficiency appears to confer protection against the development of malignancies[524, 527, 528]. The link between the overexpression of growth factors, or their receptors, and cancer is established (reviewed in [529]), and the increased concentrations of free IGF-I in obesity may play a role in this association.

4.2.8 Clinical Implications

The GH/IGF-I axis is sensitive to nutritional status, and under- or overnutrition can affect this axis at each level from regulation of secretion to intracellular signaling. Evaluating the child's nutritional status is essential in the proper interpretation of IGF-I concentrations, and failure to do this can result in misdiagnosis of a disorder of the GH/IGF-I axis.

4.2.8.1 The clinical implications of undernutrition and IGF-I measurements

IGF-I measurement has a central role in the screening evaluation of potential disorders of the GH/IGF-I axis[2]. GH stimulation testing is often used to confirm GHD in the child whose screening IGF-I concentration is low. However, these tests have poor reproducibility and specificity for this condition[130, 131].

Consequently many normal children will be misclassified as having GHD using these tests[47, 48], and this is a risk when children with low IGF-I concentrations from undernutrition undergo GH stimulation testing. These children have GH resistance and are unlikely to respond to GH therapy in the absence of nutritional replacement[484]. Ineffective GH treatment will also have significant healthcare resource implications[530], and may expose patients to risk[64, 66].

The sensitivity of the GH/IGF-I axis to transient or minor changes in nutritional substrate availability is clinically relevant in children undergoing evaluation for short stature. Brief periods of reduced caloric intake can result in reduced IGF-I concentrations, even before any effect on body composition, weight or BMI is appreciated. Thus, nutritional history should include recent, as well as chronic, dietary intake. Similarly, energy expenditure through exercise, pubertal requirements[447] or chronic disease should be considered when assessing the sufficiency of nutritional intake.

It should be remembered that the effect of nutrition on IGF-I concentration is not solely dependent on caloric intake. Inadequate protein consumption can also lower IGF-I levels. Deficiency of micro-nutrients including zinc, magnesium and iodine, as well as hormones including thyroxine (reduced in sick euthyroid syndrome during nutritional stress and/or disease) may also result in low IGF-I concentrations and should be considered as possible aetiologies.

Thus, careful evaluation of the patient's weight and BMI charts is as important as inspection of the height and height velocity charts. Three-day diet recording and analysis for macro- and micronutrient sufficiency is a worthwhile diagnostic test, as is repeating the IGF-I measurement following a period of nutritional intervention, in children suspected of undernutrition prior to performing any GH stimulation testing.

4.2.8.2 The clinical implications of overnutrition and IGF-I measurements

Unlike undernutrition, IGF-I concentrations in patients with overnutrition are less likely to result in diagnostic confusion. Total IGF-I levels are generally normal or elevated in obesity, although free IGF-I concentrations tend to be increased. Recent dietary history may be of relevance in these children, however, short-term caloric restriction in obese children may also reduce IGF-I concentrations. The effect of obesity on the GH/IGF-I axis should be considered when interpreting the results of provocative GH stimulation testing, as a blunted response to pharmacological stimuli has been reported in these children.

The long-term implications of obesity for adverse health outcomes are of significant concern in the current obesity epidemic. Nutrition-mediated alterations in the GH/IGF-I axis in obesity may play a causative role in the pathogenesis of many of these comorbidities, and is an active area of current research.

4.2.8.3 Potential new roles for IGF-I measurement

The consistent effect of undernutrition in reducing IGF-I concentrations may provide an additional clinical role to IGF-I measurement. Monitoring IGF-I concentrations has been suggested as a measure of nutritional sufficiency[436, 490, 531]. Weight gain in preterm and low birth weight infants is mirrored by increases in IGF-I levels, and this may be an additional marker of nutritional sufficiency in this population[436]. Another potential clinical role for IGF-I measurement may be the monitoring of activity of diseases associated with malabsorption, such as celiac disease[475]. IGF-I concentrations are sensitive to short-term nutritional insufficiencies, and may be affected before symptoms such as weight loss are noticeable.

4.2.9 CHAPTER CONCLUSION

IGF-I concentration is sensitive to acute and chronic changes in nutritional status. Thus, interpretation of IGF-I measurements should take into account the nutritional status of the patient. Given the poor specificity of GH stimulation

testing for GHD, caution is advised against progressing to these tests prior to addressing nutritional issues in patients where nutrition may be affecting IGF-I measurements. Likewise, the diagnosis of primary IGF deficiency cannot be made until undernutrition is excluded as a cause of the low IGF-I levels. GH and recombinant IGF-I treatment are neither appropriate nor effective interventions for increasing growth of children and adolescents with nutritional stunting. Nutritional repletion is the therapy of choice in that setting.

While weight and BMI are the most commonly used methods for describing nutritional status, noninvasive methods for studying body composition are available. In the following chapters, I will describe reference data for body composition parameters in infancy. Having described normative data for IGF-I measurement in infancy (Chapter 2.4), I will then explore the relationship between these parameters and IGF-I and –II concentrations.

CHAPTER 4.3: BODY COMPOSITION IN THE FIRST FEW DAYS OF LIFE

Publication

Hawkes CP, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Gender- and gestational age-specific body fat percentage at birth. Pediatrics. 2011;128(3):E645-E51. (Appendix L)

Presentation

Hawkes CP, O'B Hourihane, J, Kenny LC, Irvine AD, Kiely M, Murray DM. Body Composition at birth; normative values.
American Pediatric Society / Society for Pediatric Research. Denver, May 2011.
European Sociery for Paediatric Research, Newcastle, Sept 2011. (Appendix M)

4.3.1 BACKGROUND

Nutrition is a key regulator of childhood growth. This is most obvious at the extreme end of caloric undernutrition, where adolescents with anorexia nervosa are have poor linear growth that improves with weight gain[532]. Similar growth patterns are also seen in children with celiac disease before and after treatment[533-535], and in other causes of severe malnutrition[536] (See Chapter 4.2).

Assessing nutritional status in infancy can be challenging. Weight, weight-for-length, Ponderal index (mass/length^3) and body mass index (BMI, (mass/length^2) are the proxy measures of adiposity most commonly used in infants. Weight alone is an imprecise measure of adiposity, as it is comprised of all tissue compartments and is dependent on length[537]. The relationship between length and weight is age-dependent during childhood, and children with similar BMI can have different amounts of FM. Ponderal index[538] and skinfold thickness[539] are also poor predictors of body fat in early life.

The accurate measurement of FM is possible using noninvasive techniques including dual-energy x-ray absorptiometry (DXA), Air Displacement Plethysmography (ADP) and bioelectric impedance analysis (BIA)[540]. Magnetic resonance imaging (MRI)[541] and DXA can provide localised information on fat distribution. For very young infants, ADP is a preferred technique because it does not involve radiation or require sedation. This technique can also tolerate infant movement, and has been validated in infants against deuterium dilution body composition analysis[542] and DXA[543].

In this, and subsequent chapters, I will describe reference data for body composition in infancy and determine if these measures correlate with serum IGF-I and –II concentrations at birth. I will first describe the measurement of percentage body fat (%BF) at birth, before exploring and describing new indices for FM and FFM in the first three months of life.

4.3.2 AIM

The aim of this study is to describe reference data for %BF in infants born after 36 weeks' gestation within the first 4 days of life, and to determine the effect of infant sex and gestation on this.

4.3.3 METHODS

4.3.3.1 Study Population and Measurements

Infants born during the study peroid of March 2008 to October 2010 at a gestational age between 36 and 41+6 weeks' gestation, enrolled in the Cork BASELINE Birth Cohort Study, were eligible for inclusion. Eligibility and exclusion criteria for this cohort study are outlined in 2.4.3.

Gestational age, sex, birth weight and length were recorded at birth for each infant. Gestational age was determined from a first trimester scan or the last menstrual period (LMP). Gestational age based on LMP was confirmed against dates calculated from a first trimester dating scan. If there was disparity of more

than 7 days between LMP and scan dates, then the scan-based gestational age was used. Body composition was calculated using the ADP within the first four days of life.

Maternal BMI was measured on initial visit at 16 weeks gestation. Maternal cigarette use was self reported. Infant anthropometric measurements were recorded on the same day as PEAPOD™ measurement, using standardised operating procedures. Length was measured using a neonatometer to the nearest milimeter. Mid arm circumference was measured once on the left arm at the midpoint between the olecranon and acromion processes. Abdominal circumference was measured once at the level just above the umbilicus, in centimeters to one decimal place.

4.3.3.3 Air Displacement Plethysmography

The PEAPOD™ Infant Body Composition System (Life Measurement Inc, Concord, CA) is an air-displacement plethysmograph that allows for the measurement of body composition in infants between 1 and 8 kg body weight. The naked infant is placed in a closed chamber. Air displacement is measured using pressure and volume changes. Calculated body volume and body mass are used to determine body density. Age and gender-specific fat-free mass density values are used to calculate the percentage body fat[544, 545]. Interobserver variability was reduced by one trained midwife performing almost all measurements as per standard operating procedure.

4.3.3.4 Statistical analysis:

Data were entered prospectively into a secure internet database, and SPSS (version 16, SPSS Inc Chicago, Illinois USA) was used for analysis. Infants were grouped by gestational age (weeks + days) into 3 groups; 36-37^{+6}, 38-39^{+6} and 40-41^{+6}. Weighted average (Haverage) percentile values were calculated at 2.5, 5, 10, 25, 50, 75, 90, 95 and 97.5.

One way ANOVA analysis was used to compare categorical clinical and demographic variables between groups and independent samples t-tests were used to compare continuous variables. As %BF was normally distributed (Fig 1), independent samples t-tests were used to compare between groups. One way ANOVA testing was used to compare %BF between the three gestational age groups. This was also used to determine if there was a significant difference in %BF between days of PEAPOD measurement. Stepwise linear regression was used to determine the independent effect of gestation on %BF.

4.3.4 RESULTS

Of the 1203 recruited infants delivered during the study period, 743 were born between 36 to 41^{+6} weeks and had had ADP performed within the first four days of life. The excluded infants included: 417 born prior to PEAPOD availability; 31 had ADP performed after four days of life; and 12 were born outside the gestational range of 36 to 41^{+6} weeks.

Most (553/743, 74.4%) PEAPOD measurements were taken on the second or third day of life; mean (SD) = 1.9(0.9) days. Within the limit of day 0-4, day of measurement did not influence %BF (p=0.08). The demographics of our population are shown in Table 4.3.1. There was no significant difference in ethnicity, mean maternal age, mean maternal BMI and socioeconomic status between gestational age categories.

Table 4.3.1 Demographic data of study population, categorised by gestational age.
Data presented as mean (SD)
[a]New Zealand Socioeconomic Index (NZSEI-96)[546, 547]
[b]One-way ANOVA

	36-37^{+6}wk	**38-39^{+6}wk**	**40-41^{+6}wk**	**Total**	**p-value[b]**
Number	45	243	455	743	
Male, n (%)	23 (51.1)	139 (57.2)	228 (50.1)	390 (52.5)	0.2
Gestational Age, weeks	37.2 (0.6)	39.1 (0.5)	40.8 (0.5)	40.1 (1.2)	<0.001
Birth weight, g	2955 (313)	3326 (422)	3643 (437)	3498 (470)	<0.001
Age at Peapod, days	2.1 (1)	1.9 (0.9)	1.8 (0.9)	1.9 (0.9)	0.06
Caucasian Ethnicity, n (%)	44 (97.8)	240 (98.8)	447 (98.2)	731 (98.4)	0.8
Maternal age, years	29.8 (4.9)	29.7 (4.6)	29.7 (4.3)	29.7 (4.5)	0.9
Maternal University Degree or Higher, n (%)	15 (33.3)	102 (42)	222 (48.8)	339 (45.6)	0.05
Smoked in Pregnancy, n (%)	14 (31.1)	65 (26.7)	125 (27.5)	204 (27.5)	0.8
Maternal BMI at 16 weeks'	23.6 (4.2)	24.6 (4.1)	24.9 (4.1)	24.7 (4.2)	0.1
Socioeconomic Status[a] (%)					
1	2 (4.4)	10 (4.1)	34 (7.5)	46 (6.2)	0.9
2	7 (15.6)	41 (16.9)	60 (13.2)	108 (14.5)	
3	18 (40)	91 (37.4)	176 (38.7)	285 (38.4)	
4	3 (6.7)	17 (7)	37 (8.1)	57 (7.7)	
5	4 (8.9)	42 (17.3)	69 (15.2)	115 (15.5)	
6	11 (24.4)	42 (17.3)	79 (17.4)	132 (17.8)	

4.3.4.1 Gestational Age Categories

Mean body fat percentage increased with gestational age. At 36 to 37^{+6} weeks' gestation, mean (SD) %BF was 8.9% (3.5), which increased to 10.3% (4) at 38 to 39^{+6} weeks and 11.2% (4.3) at 40 to 41^{+6} weeks ($p<0.001$) (Table 4.3.2). On stepwise linear regressional analysis, gestational age remained a significant association ($R = 0.193$, $p<0.001$) when corrected for maternal BMI at 16 weeks' gestation, socio-economic group, maternal age and cigarette consumption. The other significant and consistent association with %BF on multi-variate analysis was maternal BMI at 16 weeks' gestation (Table 4.3.3). %BF increased linearly with increasing gestation and increasing maternal BMI.

Table 4.3.2: Male and Female measurements at different gestational ages
[a]Independent Sample t-test, [b]ANOVA, *Denotes variables measured by PeaPod

	Male	Female	TOTAL	P-value*
36-37^{+6} weeks gestation	23	22	45	
Birth weight g (SD)	3009 (340)	2898 (277)	2955 (313)	0.24
Fat mass g (SD)*	253 (99)	245 (112)	249 (105)	0.801
Body Fat % (SD)*	8.8 (3.2)	8.9 (3.8)	8.9 (3.5)	0.968
Fat Free Mass g (SD)*	2588 (285)	2485 (227)	2548 (261)	0.188
Fat Free Mass % (SD)*	91.2 (3.2)	91.1 (3.8)	91.1 (3.5)	0.968
Head Circumference cm (SD)	33.5 (1.3)	33.3 (1.1)	33.4 (1.2)	0.453
Ponderel Index (kg/m^3)	27 (3.1)	26 (2.5)	26.5 (2.9)	0.241
Length (cm)	48.2 (2.5)	48.2 (1.7)	48.2 (2.1)	0.973
Abdominal Circumference (cm)	32.2 (1.9)	31.3 (1.4)	31.7 (1.7)	0.085
Midarm Circumference (cm)	9.8 (0.7)	9.7 (0.8)	9.7 (0.8)	0.681
38-39^{+6} weeks gestation	139	104	243	
Birth weight	3362 (436)	3279 (399)	3326 (422)	0.13
Fat mass g (SD)*	322 (159)	351 (148)	334 (155)	0.159
Body Fat % (SD)*	9.8 (3.9)	11.1 (3.9)	10.3 (4)	0.012
Fat Free Mass g (SD)*	2879 (331)	2757 (311)	2827 (328)	0.004
Fat Free Mass % (SD)*	90.2 (3.9)	88.9 (3.9)	89.7 (4)	0.012
Head Circumference cm (SD)	34.8 (1.4)	34.1 (1.3)	34.5 (1.4)	<0.001
Ponderel Index (kg/m^3)	27.2 (2.5)	27.7 (2.3)	27.4 (2.5)	0.132
Length (cm)	49.8 (1.9)	49.1 (1.8)	49.5 (1.9)	0.004
Abdominal Circumference (cm)	33 (2)	32.9 (2)	33 (2)	0.806
Midarm Circumference (cm)	10.4 (1)	10.2 (0.9)	10.3 (1)	0.12
40-41^{+6} weeks gestation	228	227	455	
Birth weight	3687 (431)	3598 (440)	3643 (437)	0.029
Fat mass g (SD)*	358 (171)	437 (188)	397 (184)	<0.001
Body Fat % (SD)*	10 (3.9)	12.5 (4.4)	11.2 (4.3)	<0.001
Fat Free Mass g (SD)*	3122 (348)	2962 (345)	3042 (355)	<0.001
Fat Free Mass % (SD)*	90 (3.9)	87.5 (4.4)	88.8 (4.3)	<0.001
Head Circumference cm (SD)	35.4 (1.3)	34.9 (1.2)	35.1 (1.3)	<0.001
Ponderel Index (kg/m^3)	27.4 (2.5)	27.9 (2.3)	27.6 (2.4)	0.026
Length (cm)	51.2 (1.8)	50.5 (1.7)	50.9 (1.8)	<0.001
Abdominal Circumference (cm)	33.9 (1.9)	33.7 (2)	33.8 (2)	0.526
Midarm Circumference (cm)	10.8 (1)	10.8 (1)	10.8 (1)	0.397
Total cohort	390	351	743	
Birth weight	3531 (472)	3460 (466)	3498 (470)	0.04
Fat mass g (SD)*	339 (165)	400 (182)	368 (176)	<0.001
Body Fat % (SD)*	9.8 (3.9)	11.9 (4.3)	10.8 (4.2)	<0.001
Fat Free Mass g (SD)*	3003 (372)	2872 (355)	2941 (370)	<0.001
Fat Free Mass % (SD)*	90.2 (3.9)	88.1 (4.3)	89.2 (4.2)	<0.001
Head Circumference cm (SD)	35.1 (1.4)	34.6 (1.3)	34.8 (1.4)	<0.001
Ponderel Index (kg/m^3)	27.3 (2.5)	27.7 (2.4)	27.5 (2.5)	0.023
Length (cm)	50.5 (2)	50 (1.9)	50.3 (2)	<0.001
Abdominal Circumference (cm)	33.4 (2)	33.3 (2.1)	33.4 (2)	0.503
Midarm Circumference (cm)	10.6 (1)	10.6 (1)	10.6 (1)	0.911

Table 4.3.3: Stepwise linear regression of factors affecting body fat percentage.
*Maternal BMI at 16 weeks gestation,
** Number of cigarettes smoked per day during pregnancy as measured by maternal report.

Independent variables	Correlation coefficent	p-value	Standardised Beta coefficient	t-value
Gestational age	0.200	<0.001	0.192	**5.32**
Maternal BMI*	0.114	0.006	0.099	**2.71**
Cigarette consumption**	-0.011	0.387	-0.011	**-0.302**
Socio-economic group	0.029	0.450	0.013	**0.329**
Maternal age	-0.24	0.263	-0.026	**-0.672**
Dependent variable = %Body fat day 1-4. F =7.815, p<0.001				

4.3.4.2 Effect of gender

The %BF in males and females was normally distributed within each gestational age category (Fig 4.3.1). Males had lower mean %BF than females in each category. The difference became more pronounced with advancing gestational age, and was statistical significance in the 38 to 39^{+6} (p=0.012) and 40 to 41^{+6} (p<0.001) weeks' gestation categories.

While females had a greater %BF than males at each gestational age, males had a greater birth weight. This was not significant at 36-37^{+6} weeks (p=0.24) or 38-39^{+6} weeks (p=0.13), but was significance at 40-41^{+6} weeks; with males weighing 3683g (SD=435g) and females weighing 3593g (SD=447g) (p=0.029). It should be noted that there were increased numbers of subjects at later gestational ages, which may have increased power to detect differences.

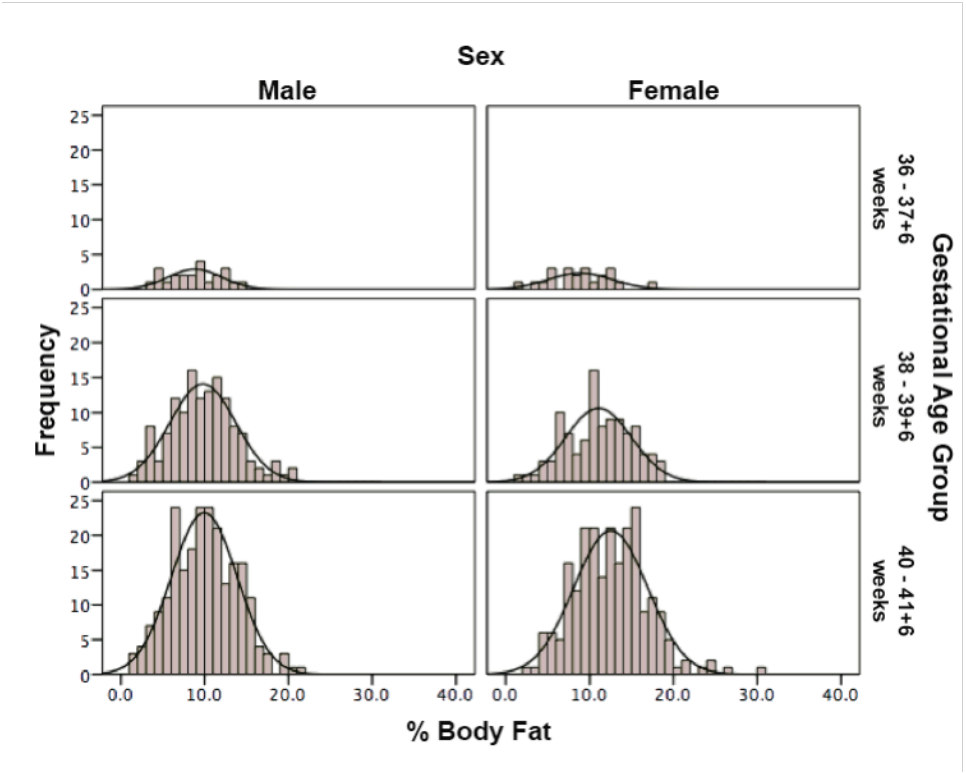

Figure 4.3.1 Body fat percentages for males and females at 36-37^{+6}, 38-39^{+6} and 40-41^{+6} weeks gestation. Normal curves displayed.

4.3.4.3 Centile Chart

A centile chart was compiled for males, females and all infants at each gestational age category, and is shown in Table 4.3.4.

Table 4.3.4: Centiles for body fat percentage according to gestational age and sex

Centile	**36-37^{+6} weeks**			**38-39^{+6} weeks**			**40-41^{+6} weeks**		
	Male	Female	All	Male	Female	All	Male	Female	All
97.5th	14.9	17.5	17.1	19	18.2	18.4	18.2	22.1	19.8
95th	14.5	16.9	14.4	17.1	17.7	17.5	16.2	19.2	18.3
90th	13	13.1	12.9	14.5	16.3	15.5	15	17.9	16.7
75th	11.9	12	11.9	12.2	14.1	13	12.7	15.4	14.2
50th	9.2	8.9	9.2	9.6	11	10.3	9.9	12.5	10.9
25th	6	5.7	5.9	7.2	7.9	7.5	6.9	9.4	8.1
10th	4.6	4	4.4	4.7	6.2	5.1	4.9	7.2	5.8
5th	3.4	1.8	3.3	3.2	4.7	3.4	3.4	5.6	4.4
2.5th	3.1	1.4	1.7	2.4	2.9	2.6	2.8	4.7	3.2

4.3.5 DISCUSSION

This large observational birth cohort study shows the distribution of %BF in the first 4 days of life amongst first born infants greater than 36 weeks' gestation in a largely Caucasian Irish population. We have shown an upward trend in %BF at increasing gestational age, and demonstrated a significantly higher %BF at birth in females than in males. We have created a centile chart for %BF in male and female infants that will assist physicians and researchers in the interpretation of measured neonatal body fat percentage.

Previous studies of %BF at birth in term infants have shown varying mean values. This has varied from 8.6±3.7% (n=87) in Italian infants[548], to 10.6 ± 4.6% (n=87) in cohort of 87 American infants[549] and 12.9 ± 4% in 108 full term Swedish infants in the first 10 days of life[550]. No previously studied cohorts have been large enough to delineate normative data for gestational age categories in term infants. Our mean values varied considerably depending on the gestational age and sex of our studied infants and this may explain the variance seen between previous reports.

In this cohort, we have found that females have a greater %BF than males at birth at each of the studied gestational age categories; a difference that increased with advancing gestational age. While it is known that female children[551] and adults[552] have higher fat mass and lower lean body mass than males, there is disagreement in the published literature regarding the degree of difference, and whether or not this is present from birth. In 1967, Foman et al first observed this difference using a multicomponent model to determine body fat, based on measurements of total body water, total body potassium and bone mineral content. This finding has been replicated using dual energy x-ray absorptiometry[553, 554], and ADP[555]. However, Butte used the multicompartment model in 76 infants and did not find a difference between sexes at 2 weeks of age[556]. Eriksson and Gilchrist in two seperate studies using ADP found that %BF did not differ significantly between sexes at one and two weeks of age[550]. Once again these cohort sizes were much smaller (108 and 80 infants respectively).

As expected we found that male infants were heavier than their female counterparts at each gestational age. Despite this, their %BF was lower, meaning that this increase in weight was due to increased fat free mass. There is evidence that boys grow faster than girls in utero, and are more reliant on placental function and maternal nutrition during pregnancy[557, 558]. Male infants seem more vulnerable to undernutrition, as evidenced by the greater effect of the Dutch famine on the male risk of later cardiovascular disease[559], and the greater effect of malnutrition on male infants in animal experiments[560, 561].

As our study recruited primiparous volunteers with singleton pregnancies, and took place in a single Irish centre, there is a potential bias that may affect the generalisability of the results. However, our study population closely reflects that of the Irish population as a whole. In the Irish census of 2006, the demographics of women aged 15-44 compared with our study population were as follows: Caucasion 94% vs 98.4%; Completed 3rd level education 33.7% vs 45.6%[562]. A recent study of 1000 pregnant Irish women recorded a mean(SD) first trimester BMI equal to 25.7[563]. This compares closely with 24.7(4.2) found in our study population. Thus, the babies included in our study are close to a representative sample of Irish first born infants.

4.3.6 CHAPTER CONCLUSION

Neonatal adiposity cannot be evaluated without accurate reference data, and this study was an important first step in characterising this. The observation that males weigh more but have less body fat than females demonstrates that body composition measurement may provide additional nutritional information beyond weight in early life. While %BF is commonly used as a measure of adiposity, it does not correct sufficiently for body size. In the next study, we build on the data generated from this study by developing a measure of fetal adiposity that is independent of infant size and can be used in describing body composition in the first three months of life.

CHAPTER 4.4: DEFINING BODY COMPOSITION IN THE FIRST TWO MONTHS OF LIFE AND CORRELATING WITH GROWTH AT TWO YEARS

Publication

Hawkes CP, Zemel BS, Kiely M, Irvine AD, Kenny LC, O'B Hourihane J, Murray DM. Body composition within the first 3 months: optimized correction for length and correlation with BMI at 2 years. Horm Res Paediatr. 2016;86(3):178-187 (Appendix N).

Presentation

Hawkes CP, Zemel BS, Kiely M, Irvine A, Kenny LC, O'B Hourihane J, Murray DM. Body composition in the first 2 months of life – optimized correction for length, reference data and correlation with obesity at 2 years. American Pediatric Society / Society for Pediatric Research. Baltimore, May 2016 (Poster Presentation) (Appendix O).

4.4.1 INTRODUCTION

In the previous study, I described reference data for %BF at birth. However, a confounding effect of body size on total FM and FFM has been described in adults[564] and children[565]. %BF is also susceptible to this effect, as two individuals with the same %BF could have very different amounts of absolute FM[566]. Thus, the determination of an index of FM and FFM that is independent of size was required in order to investigate the interaction of body composition with IGF-I and –II concentrations (Chapter 4.5).

FM relative to length (L) is often used to correct FM for body size; fat mass index (FM/L^2) and fat free mass index (FFM/L^2) are commonly used in older children and adults. Whether these represent the optimal indices to correct FM and FFM for length in infancy has not been examined previously.

Rapid weight gain in early infancy increases future cardiometabolic risk[567-570] and predicts obesity in later childhood[571, 572], adolescence and adulthood[573, 574]. In addition to being a marker of nutritional status, I hypothesised that changes in body composition in infancy may also identify children who are at risk of future obesity.

4.4.2 AIM

The aims of this study were 1) to determine the optimal index of FM and FFM that is independent of length in the first three months of life, 2) to describe reference data for FM, FFM, %BF, as well as for parameters corrected for length in early infancy and 3) to determine if these measurements in early infancy can be used to identify children who will have an increased BMI at two years of age.

4.4.3 METHODS

Mothers and their children were recruited at 20 weeks' gestation through the SCOPE pregnancy study[575] and followed up from birth through the Cork BASELINE birth cohort study (ClinicalTrials.gov NCT: 01498965) (additional details in 2.4.3)[344]. Infants were recruited over the study period from August 2008 to August 2011. Ethical approval was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

For this study, we included all children born between 37 weeks and 41 weeks, 6 days' gestation. Birth assessments of body composition were defined as measurements obtained up to and including day four of life. Ninety percent of two month visits in this cohort took place between 49 and 86 days of life, and these were included as the "two month" body composition evaluations.

4.4.3.1 Demographics

Details regarding feeding type were recorded at each visit from parental report. Gestational age was determined using the first day of the last menstrual period

(LMP). Ultrasound gestational age was used if it was performed before 16 weeks' gestation and a discrepancy of greater than six days between this and LMP was noted, or if the ultrasound scan at 20 weeks' reported a discrepancy of 10 or more days with LMP.[575]

4.4.3.2 Anthropometry

At birth and two months, weight, length, and occipito-frontal circumference (OFC) were measured using standardised protocols and recorded on a secure online database. Supine length was measured to the nearest millimeter at birth using a Harpenden neonatometer (Harlow Healthcare, London, UK), and at two months using a Seca 210 mat (Harlow Healthcare, London, UK). Standing height was measured at 24 months of age using a Seca 206 tape (Harlow Healthcare, London, UK). Infants were weighed unclothed.

4.4.3.3 Body composition assessment

Body composition was measured at birth and two months of age using ADP (PEAPOD^{TM} Infant Body Composition System, Life Measurement Inc, Concord, CA)[576], as described in 4.3.4.2.

4.4.3.4 Statistical Analysis

All data were analyzed using SPSS version 21.0 (IBM, New York, USA). Mean (SD) were reported for normally distributed data, and comparison between independent groups were made using Student's t-test for independent variables. Categorical variables (exclusive breastfeeding status and Caucasian ethnicity) were compared using Chi Squared tests.

Determination of the optimal length-corrected index of fat mass and fat free mass in infancy

In order to determine the optimal index of FM and FFM corrected for length, we used a method previously described by Wells *et al*[565], and Cole *et al*[577]. Logarithmic conversion of FM (LogFM), FFM (LogFFM) and Length

(LogLength) at birth and two months were calculated. Linear regressions of LogFM or LogFFM were performed separately with LogLength as the independent variable, similar to the Benn Index used to define the optimal power for weight-for-height indices[578]. This analysis used measurements at birth and aged two months. The slope of the regression line for each analysis delineates the optimal power of length in the FM/L^x or FFM/L^y that is least dependent on length.

We confirmed these results using the closest integer of x or y determined by the previous calculation. Linear regression for length (independent variable) and FM/L^x (dependent variable) were performed. A regression coefficient close to 0 between length and FM/L^x was considered to represent an index of fatness that is independent of length. Pearson's correlation coefficient was also used to determine the contribution of length to the measure of FM/L^x. This analysis was performed separately at birth and at two months. The same approach was used for FFM.

Reference values of body fat percentage, fat mass/length^3 and fat free mass/length^2 at birth and two months

Gender-specific reference curves for %BF, FM/L^3 and FFM/L^2 were generated using LMS Chartmaker Pro (Harlow Printing Ltd., Tyne and Wear, UK). The LMS method used by this software has been described previously[347, 348]. A Box-Cox transformation is used to obtain normality, and separate smooth curves are generated for skewness, median and variability. These are constrained to smooth changes over time, and combined in one graph.

Evaluation of body composition at birth and two months, and BMI at two years

Children with ADP performed at birth and two months, and who had BMI available at two years were included in this analysis. Age- and sex-specific z-scores for FM, FFM, FM/L^3, FFM/L^2, and %BF were generated from this population using the reference values described above. Sex-specific BMI z-

scores at two years of age based on the World Health Organization reference[27] were calculated using Stata 12.0 (StataCorp, College Station, TX, USA),[346]. Multiple linear regression analysis was performed using BMI z-score at two years as the dependent variable and age- and sex-specific z-scores for FM/L^3, FFM/L^2 at birth and at two months as the independent variables. Logistic regression was used to determine the odds of having a BMI z-score ≥ 2 at two years is based upon FM, FFM, FM/L^3, FFM/L^2 and %BF z-scores at birth and two months.

4.4.4 RESULTS

Of the 2137 infants included in this birth cohort, 2032 were born between 37 weeks and 41 weeks, 6 days' gestation. Body composition was measured using ADP before five days of age in 1063 (526 females) and from 49 to 86 days of life in 922 (445 females). ADP was performed in 725 term children (348 female) both at birth and two months of age. BMI at two years was available in 562 (267 female) of these infants (Figure 4.4.1). Characteristics of the infants who had ADP body composition assessment performed at birth and two months of age are shown in Table 4.4.1.

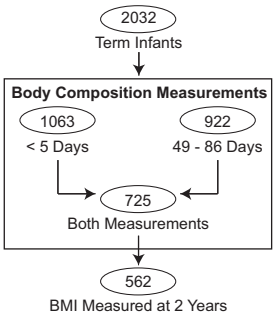

Figure 4.4.1: The number of subjects recruited and undergoing body composition measurement at birth (<5 days) and two months (49-86 days), and body mass index measurement at two years.

Table 4.4.1: Body Composition Measurements within the first four days of life in term infants. Mean (SD).
P values represent the significance of the difference between males and females in each parameter at each time point measured using t-test (or *Fisher's exact test, 2-sided).

	BIRTH (0-4 days)			**TWO MONTHS (49-86 days)**		
	Male (n=537)	**Female (n=526)**	**p**	**Male (n=477)**	**Female (n=445)**	**p**
Gestation, weeks	40.2 (1.1)	40.2 (1.1)	0.5	40.2 (1.1)	40.2 (1.1)	0.83

Exclusively Breastfed at time of measurement, n (%)	158 (29.4%)	187 (35.6%)	0.3*	131 (27.5%)	132 (31.9%)	0.47*
Caucasian, n (%)	531 (98.9%)	517 (98.5%)	0.81	531 (98.9%)	517 (98.3%)	0.17
Weight, kg	3.57 (0.45)	3.47 (0.45)	0.001	5.65 (0.69)	5.25 (0.57)	<0.001
Length, cm	50.7 (2)	50.1 (1.9)	<0.001	59 (2.1)	57.9 (2.2)	<0.001
Head Circumference, cm	35.1 (1.4)	34.5 (1.3)	<0.001	40.2 (1.3)	39.3 (1.4)	<0.001
Age at PeaPod, days	1.9 (1)	1.78 (1)	0.045	65.5 (8)	65.7 (8.2)	0.7
Total body mass, kg	3.38 (0.44)	3.29 (0.42)	0.001	5.65 (0.68)	5.25 (0.57)	<0.001
Fat Mass, kg	0.36 (0.17)	0.41 (0.17)	<0.001	1.21 (0.34)	1.19 (0.31)	0.18
Fat free mass, kg	3.02 (0.35)	2.88 (0.33)	<0.001	4.44 (0.45)	4.06 (0.38)	<0.001
Body fat, %	10.2 (4)	12.04 (4)	<0.001	21.2 (4.3)	22.3 (4.2)	<0.001
Fat Free Mass Index, kg/m^2	11.74 (1.01)	11.46 (0.98)	<0.001	12.73 (0.84)	12.1 (0.8)	<0.001
Fat Mass/$length^3$, kg/m^3	2.67 (1.18)	3.18 (1.17)	<0.001	5.88 (1.52)	6.07 (1.4)	0.046

Determination of the optimal length-corrected measure of fat mass in infancy

We first determined the optimal index of FM, and FFM least influenced by length. The overall slope of the regression line relating LogFM to LogLength at birth was 5.3 (5.9 males, 5.6 females), and at two months was 3.3 (3.5 males, 3.4 females). This indicates that FM/L^5 at birth and FM/L^3 at two months are optimal indices of fatness corrected for length. The overall slope of the regression line for LogFFM with LogLength was 2 at birth (1.9 males, 2.1 females) and 2.2 at two months (2.2 males, 1.9 females).

Separate regression analyses were performed to confirm that these indices minimised the association of FM and FFM with length. The regression coefficient for FM/L^3 with length was 0.12 (SEM 0.02) (R^2 0.04) at birth, and -0.005 (SEM 0.03) (R^2<0.001) at two months (Table 2). The regression coefficient for FFM/L^2 was 0.03 (SEM 0.02) (R^2 0.003) at birth, and 0.04 (SEM 0.01) (R^2 0.01) at two months. These results indicate that FM/L^3 minimises the

association of FM and length at birth and two months. While FM/L^5 may be more independent of length at birth, the contribution of length to the FM/L^3 at birth is low (R^2 = 0.04), and using the same index as two months allows for comparison between age groups. Using a similar analysis (Table 4.4.2), FFM/L^2 should be used as an index of FFM at birth and two months.

Table 4.4.2: Regression analysis of length on FM and FM/L^x at birth and two months of age.

	0-4 days of life (n=1063)			**49 – 86 days of life (n=922)**		
	R^2	**Regression Coefficient (SEM) for Length**	**p**	**R^2**	**Regression Coefficient (SEM) for Length**	**p**
Dependent Variable						
FM	0.194	0.039 (0.002)	<0.001	0.18	0.062 (0.004)	<0.001
FM/L	0.136	0.061 (0.005)	<0.001	0.076	0.066 (0.008)	<0.001
FM/L^2	0.083	0.091 (0.009)	<0.001	0.019	0.055 (0.014)	<0.001
FM/L^3	0.039	0.121 (0.018)	<0.001	<0.001	-0.005 (0.025)	0.84
FM/L^4	0.011	0.123 (0.036)	0.001	0.024	-0.18 (0.042)	<0.001
FM/L^5	<0.001	0.01 (0.072)	0.9	<0.001	0.004 (0.015)	0.8
FFM	0.531	0.124 (0.004)	<0.001	0.572	0.156 (0.004)	<0.001
FFM/L	0.214	0.129 (0.007)	<0.001	0.3	0.149 (0.007)	<0.001
FFM/L^2	0.003	0.029 (0.015)	0.05	0.012	0.043 (0.012)	<0.001
FFM/L^3	0.144	-0.401 (0.029)	<0.001	0.161	-0.293 (0.021)	<0.001
FFM/L^4	0.437	-1.719 (0.058)	<0.001	0.494	-1.141 (0.036)	<0.001
FFM/L^5	0.644	-5.285 (0.117)	<0.001	0.698	-3.072 (0.063)	<0.001

Reference values for adjusted fat mass in infancy at birth and two months of age

Reference centile charts for FM/L^3, FM and %BF are shown in Figure 4.4.2, and FFM/L^2 and FFM are shown in Figure 4.4.3. During the first two months of life, mean (SD) FM/L^3 increased approximately two-fold (males 2.7 (1.2) to 5.9 (1.5) kg/m^3, females 3.2 (1.2) to 6.1 (1.4) kg/m^3). A similar increase is noted in %BF

from (males 10.2 (4) to 21.2 (4.3)%, females 12 (4) to 22.3 (4.2)%). FFM/L^2 remained relatively constant during these two months (males 11.7 (1) to 12.7 (0.8) kg/m^2, females 11.5 to 12.1 (0.8) kg/m^2). Females had increased FM/L^3 and %BF at birth ($p<0.001$, $p<0.001$) and two months ($p=0.046$, $p<0.001$), and lower FFM/L^2 at birth ($p<0.001$) and two months ($p<0.001$) compared to males (Table 4.4.1).

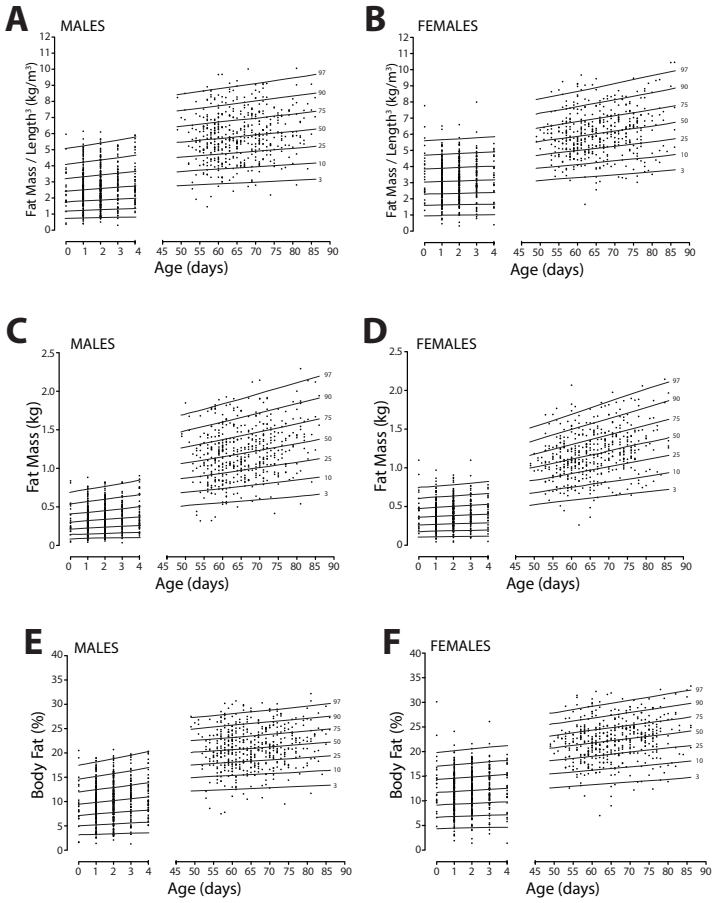

Figure 4.4.2: Sex-specific centile charts for fat mass/length^3 (A and B), fat mass (C and D) and body fat percentage (E and F) in term infants. Note that these charts show normative data from days 0 to 4, and 49 to 86 days in this population.

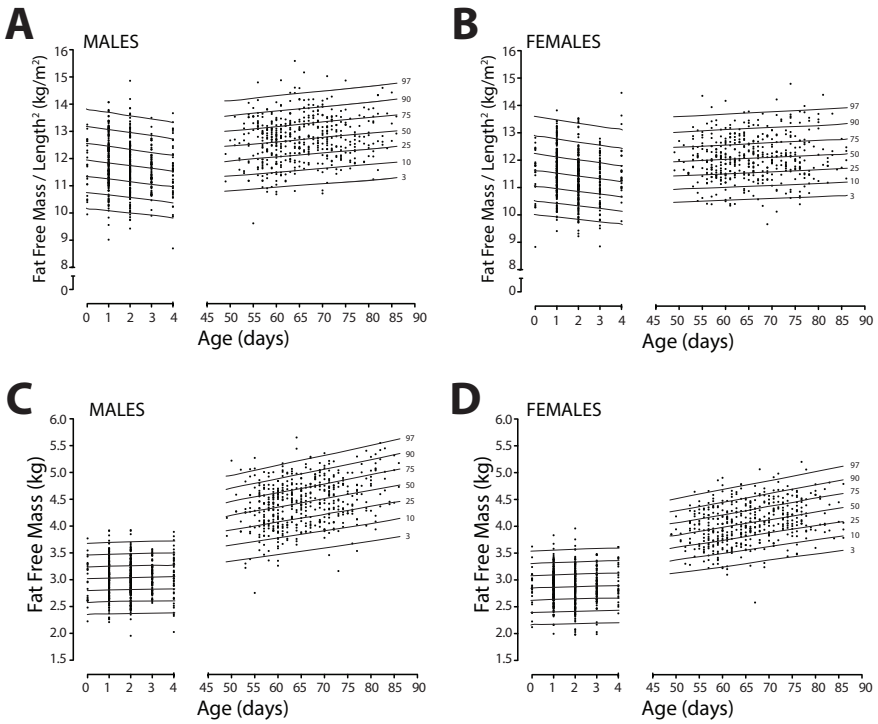

Figure 4.4.3: Sex-specific centile charts for fat free mass (A and B), and fat free mass/length^2 in term infants (C and D). Note that these charts show normative data from days 0 to 4, and 49 to 86 days in this population.

Body composition and anthropometric determinants of increased BMI at two years of age

Complete data on body composition measurements at birth and two months and BMI at two years were available in 562 (267 female) participants. The mean (SD) BMI at two years was 16.6 (1.5) in males, and 16.4 (1.3) in females. Mean (SD) BMI z-score at two years for males and females was 0.64 (0.9) and 0.67 (0.9) respectively.

FM and FFM at birth explained 0.06 of the variance in BMI z-score at age two years. By two months of age, these measures explained up to 0.18 of this variance (Table 4.4.3). Correcting FM and FFM for length did not improve the overall explained variance in the linear regression model. Changes in body composition z-scores were also not strong predictors of BMI z-score at age two years. Weight and length z-scores at two months were marginally better at predicting BMI z-score at two years than body composition at two months

(R^2=0.21). Weight z-score at two months was the strongest independent predictor of BMI at two years (R^2=0.17), and compares favourably with FM/L^3 (R^2=0.02 at birth, 0.14 at two months) or FFM/L^2 (R^2=0.06 at birth, 0.11 at two months).

Table 4.4.3: Linear regression models for BMI z-score at two years.
Models were generated using FM and FFM corrected for length, or not corrected for length. The predictive value of standard weight and length measurements were also used in a separate model. Explained variance (R^2) of models using single variables at one timepoint [a], at two variables at one timepoint [b] or two variables at two timepoints [c] are presented separately. z-scores are sex- and age-specific.

BMI z-score at 2 years	Estimate	SEM	95% CI	P value	R^2		
Corrected for Length: FM/L^3 and FFM/L^2 at birth and 2 months, for BMI at 2 years							
FFM/L^2 z-score at birth	0.107	0.037	0.035, 0.18	<0.001	0.06 [a]	0.07 [b]	0.19 [c]
FM/L^3 z-score at birth	0.027	0.034	-0.041, 0.094	0.4	0.02 [a]		
FFM/L^2 z-score at 2 months	0.179	0.038	0.104, 0.253	<0.001	0.1 [a]	0.17 [b]	
FM/L^3 z-score at 2 months	0.241	0.035	0.172, 0.31	<0.001	0.11 [a]		
Not Corrected for Length: FM and FFM at birth and 2 months, for BMI at 2 years							
Fat Free Mass z-score at birth	-0.110	0.056	-0.12, 0.099	0.845	0.06 [a]	0.06 [b]	0.18 [c]
Fat Mass z-score at birth	0.001	0.038	-0.074, 0.076	0.981	0.03 [a]		
Fat Free Mass z-score at 2 months	0.201	0.053	0.096, 0.306	<0.001	0.11 [a]	0.18 [b]	
Fat Mass z-score at 2 months	0.258	0.039	0.182, 0.333	<0.001	0.14 [a]		
Weight and Length at birth and two months, for BMI at 2 years							
Weight z-score at Birth	0.128	0.059	0.013, 0.244	0.03	0.06 [a]	0.08 [b]	0.22 [c]
Length z-score at Birth	-0.130	0.052	-0.233, -0.028	0.013	0.01 [a]		
Weight z-score at 2 Months	0.547	0.056	0.436, 0.657	<0.001	0.17 [a]	0.21 [b]	
Length z-score at 2 Months	-0.169	0.053	-0.273, -0.065	0.002	0.03 [a]		

At two years of age, 39 of these children (23 males, 16 females) had BMI z-scores ≥ 2. Increasing z-scores for FM and FFM at birth and two months increased the odds of having a BMI ≥ 2 at two years of age. Increases in FM,

FM/L^3 and weight z-scores at two months of age were associated with the largest increase in the odds of having an increased BMI at two years (Table 4.4.4).

Table 4.4.4: The odds ratio (95% CI) of having BMI z-score ≥ 2 at two years for every unit increase in z-score of body composition measurements at birth and two months of age.
FFM=Fat Free Mass, FM=Fat Mass, L=Length

BMI z-score ≥ 2, at 2 years	**Odds Ratio**	**95% CI**	**P**
FFM/L^2 z-score at birth	1.36	1.02, 1.82	0.036
FM/L^3 z-score at birth	1.46	1.09, 1.96	0.012
FFM/L^2 z-score at 2 months	1.62	1.21, 2.16	0.001
FM/L^3 z-score at 2 months	2.5	1.83, 3.42	<0.001
Body Fat % z-score at birth	1.38	1.03, 1.85	0.032
Body Fat % z-score at 2 months	2.24	1.64, 3.04	<0.001
Δ FFM/L^2 z-score (birth to 2 months)	1.01	0.74, 1.38	0.95
Δ FM/L^3 z-score (birth to 2 months)	1.3	0.99, 1.7	0.06
Δ Fat Mass z-score (birth to 2 months)	1.36	1.03, 1.8	0.032
Δ Fat Free Mass z-score (birth to 2 months)	1.07	0.66, 1.71	0.79
Δ Body Fat % z-score (birth to 2 months)	1.28	0.98, 1.69	0.075
Δ Body Weight z-score (birth to 2 months)	0.88	0.54, 1.44	0.6
Fat Free Mass z-score at birth	1.58	1.16, 2.15	0.004
Fat Mass z-score at birth	1.52	1.13, 2.04	0.005
Fat Free Mass z-score at 2 months	1.79	1.33, 2.41	<0.001
Fat Mass z-score at 2 months	2.71	1.97, 3.72	<0.001
Weight z-score at Birth	1.9	1.38, 2.63	<0.001
Length z-score at Birth	1.34	1, 1.78	0.048
Weight z-score at 2 Months	2.27	1.64, 3.13	<0.001
Length z-score at 2 Months	1.31	0.98, 1.75	0.072

4.4.5 DISCUSSION

Here I describe reference data for body composition measured by ADP at birth and two months of age in a large cohort of infants. FM and FFM are influenced by length at birth and two months, and these analyses provide optimal indices for length that minimise this interaction. This study also describes reference charts for these new measures, which allow for the description of FM and FFM independent of length.

Infants undergo a significant change in body composition over the first two months. There is a two-fold increase in mean FM/L^3 and %BF, while FFM/L^2

remains relatively constant. This demonstrates that, while the absolute FFM increases, it remains fairly constant relative to linear growth. In contrast, there is a disproportionate increase in FM. This increase in FM in the first two months of life has also been shown in other smaller studies[579, 580] and the rate of increase in FM slows after this first two months[579]. A doubling of adiposity in healthy subjects, as demonstrated by FM/L^3 or %BF in this study, has not been reported at any other period in postnatal life[581].

Rapid weight gain in early life may have significant implications for future health, particularly cardiometabolic risk[568, 582-584], but it is not known if these associations are related to specific changes in body composition. It is possible that the reference charts provided in this manuscript may be used to determine if an infant's FM/L^3 or FFM/L^2 is within the normal ranges. However, the clinical implications of FM/L^3 or FFM/L^2 outside of the normal range in the first two months of life are currently unknown and subject to further study. These parameters may be of particular relevance for monitoring children born small for gestational age (SGA)[585], where rapid weight gain in the first two years is associated with increased abdominal fat and adiposity as well as reduced lean mass at four years of age[586]. Infants born SGA have decreased insulin sensitivity within the first two years of life, [586, 587] and this decreases over the following years in association with the rate of early weight gain[587]. It is possible that tracking changes in FM/L^3 or FFM/L^2 in these infants may represent an area for future research and intervention to decrease future cardiometabolic risk.

In this study, we have investigated the utility of body composition measurements in the first two months in predicting obesity at two years. There is no accepted definition of obesity at two years of age, and it is possible that BMI at this age is not an ideal indicator of future cardiometabolic risk. We have shown that weight and length measurements at two months confer greater prediction of elevated BMI at two years than body composition measurements at two months, which may reflect a relatively constant pattern in weight and linear growth between two months and two years. The limited utility of BMI in early life is also shown by

studies that demonstrate BMI at one and two years of age being weaker predictors of obesity in adulthood, than BMI in later childhood and adolescence[573, 588].

Limitations in the utility of the reference data described in this study are related to the unknown effects of ethnic and societal influences on body composition during the first two months of life. This Irish birth cohort study includes a relatively homogeneous, stable population of infants of European ancestry born in a single hospital in one mixed urban/rural area in Ireland. In this population, the rate of exclusive breastfeeding at two months was low in comparison to many other populations, at approximately 30%. Feeding modality may affect body composition at this early age, and exclusively formula fed infants have been described to have increased FFM, but not necessarily FM, when compared with exclusively breastfed infants at four months[580]. These factors may limit the generalisability of these reference data.

In conclusion, there are significant changes in adiposity in the first two months of life whereas FFM remains relatively constant when corrected for length. Although the clinical implication of this change in body composition is unknown, significant weight gain at this age confers increased future cardiometabolic risk and requires further investigation. The length correction indices, and reference data provided in this study contribute to our understanding of the changes in body composition occurring during this time, but do not increase our prediction of BMI at two years above standard weight and length measurements.

4.4.6 Chapter Conclusion

In Chapters 4.3 and 4.4, I have explored the use of ADP in describing body composition in infancy. I initially described and published reference data for %BF at birth, but subsequently developed new measures of reporting FM and FFM corrected for infant size. Given the relationship between nutritional status

and the GH / IGF axis (Chapter 4.2), I will investigate the relationship between these body composition indices and IGF-I and IGF-II production in the next chapter.

CHAPTER 4.5: CORRELATION OF INSULIN-LIKE GROWTH FACTOR-I AND –II WITH BODY COMPOSITION

Presentation

Hawkes CP, Zemel BS, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life. Pediatric Endocrine Society, September 2017 (Poster Presentation) (Appendix P).

Manuscript under review

Hawkes CP, Zemel BS, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life.

4.5.1 INTRODUCTION

In this chapter, I will bring together data and concepts from a number of previous chapters. Having reviewed the interaction between the GH / IGF-I axis and nutritional status (Chapter 4.2), described body composition in the first few days of life (Chapter 4.3), and developed a new size-independent index of FM and FFM in the first two months of life (Chapter 4.4), I will now determine if there is a relationship between body composition over the first two months and IGF-I and –II concentrations at birth. I will use the new IGF-I and –II measurements by LCMS described in Chapter 2.4 to explore this relationship.

4.5.2 BACKGROUND

IGF-I and –II play an important role in regulating fetal growth, as demonstrated by infants with IGF-I and –II signaling defects having significant prenatal growth failure[152, 329]. Regulation of IGF-I and -II *in utero* appears to be more

dependent on nutrition[196] than GH. Infants with GHD have normal size at birth[332, 589] whereas IGF-I and –II concentrations are reduced in intrauterine growth restriction[590], and animal models show that IGF-I and –II expression are reduced in response to intrauterine nutritional deprivation[591, 592]. In early infancy, nutrition continues to play a major role in IGF-I production where formula feeding and high protein intake are associated with increased IGF-I concentrations[299, 313, 437].

Birth weight is often used as a proxy for adiposity, but these do not always correlate[269, 270] and it is possible that IGF-I and –II concentrations are associated more closely with body composition than birth weight. Infants born SGA have a greater deficit in body fat than lean mass and have lower IGF-I concentrations[267], suggesting that IGF-I levels may be more closely associated with body fat than lean mass at birth. Similarly, IGF-I levels at birth are associated with Ponderal index, a marker of adiposity[221]. At five years, IGF-I concentrations correlate more closely with FFM whereas IGF-II concentrations are associated with FM[593]. At eight years of age, IGF-I concentrations continue to correlate more closely with FFM[594, 595], but may also be associated with adiposity[594]. These associations of IGF-I and –II with body composition parameters diminish during puberty[595].

IGF-I measurement may also be useful in assessing growth trajectories, but whether this can be used to predict changes in body composition is not known. In children born appropriate for gestational age, higher IGF-I concentrations at three months of age are associated with increased weight gain over the preceding three months[299]. Higher IGF-I concentrations at three months of age are also associated with increased linear growth over the following nine months, without an associated increase in adiposity[301]. This dynamic relationship between IGF-I concentrations and rate of growth is also seen at five years of age, where higher IGF-I concentrations are seen in children who had more weight gain over the first two years of life regardless of weight at the time of IGF-I measurement[596].

There are limited data describing the association of IGF-I and -II concentrations with detailed body composition measurements at birth, or studying the predictive value of IGF-I and –II concentrations on subsequent dynamic changes on body composition. The aim of this study is to determine if IGF-I and -II measurements at birth are associated with body composition at birth, and the trajectory of body composition changes in the first two months of postnatal life.

4.5.3 METHODS

Children were enrolled in the Cork BASELINE birth cohort study at birth (ClinicalTrials.gov NCT: 01498965) between August 2008 and August 2011[344] (additional details in 2.4.3). Ethical approval was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

4.5.3.1 Body Composition assessment

ADP (PEAPOD^{TM} Infant Body Composition System, Life Measurement Inc, Concord, CA)[576] was used to measure body composition at birth and two months, as described in 4.3.4.2.

Birth measurements were included if they occurred within the first four days of life and two month measurements if they were taken between 49 and 86 days of life. In order to adjust FM and FFM for body size, FM/length^3 (FM/L^3) and FFM/L^2 were calculated and converted to age- and sex-specific z-scores (Chapter 4.4)[270].

4.5.3.2 Sample collection and storage

Umbilical cord samples were collected at birth. They were processed to serum within three hours of collection, and stored at -80^{o}C until analysed. Samples were analysed at Quest Laboratories (NJ, USA) by LCMS, as outlined in 2.4.3.5.

4.5.3.3 Statistical Analysis

The LCMS assay cannot detect IGF-I concentrations below 16 ng/ml or IGF-II concentrations below 32 ng/ml. Where samples had concentrations below these values, a concentration of 15 ng/ml for IGF-I or 31 ng/ml for IGF-II was assigned. Z-scores for FM/L^3 and FFM/L^2 were age- and sex-specific at birth and two months. The change in z-score between birth and two months was calculated by subtracting the age- and sex-specific z-score at birth from the two-month z-score.

Data analyses were performed using SPSS 21.0 (IBM, New York, USA). Mean (SD) were reported for normally distributed data and compared using Student's independent sample T-tests. Linear regression analysis was used to evaluate the relationship between continuous variables.

4.5.4 RESULTS

There were 2137 infants enrolled in the Cork BASELINE birth cohort. Of these, 105 were excluded from this study for prematurity, 932 did not have sufficient cord blood available for IGF measurement, and a further 499 did not have body composition measurement performed both at birth and two months. Complete measurements of IGF-I and –II at birth and ADP measurements at birth and two months of age were available in 601 term infants (317 male). Characteristics of these subjects are shown in Table 4.5.1.

Table 4.5.1: Characteristics of population. Unless otherwise stated, mean(sd) are presented.

	Males	**Females**	**All**
Number, n	317	284	601
Gestational Age, weeks	40.2 (1.1)	40.2 (1)	40.2 (1.1)
Birth weight, kg	3.57 (0.45)	3.49 (0.45)	3.53 (0.45)
Weight at 2 months, kg	5.66 (0.68)	5.3 (0.57)	5.49 (0.66)
Length at birth, m	0.51 (0.02)	0.5 (0.02)	0.5 (0.02)
Length at 2 months, m	0.59 (0.02)	0.58 (0.02)	0.59 (0.02)
IGF-I, ng/ml	49.6 (23.9)	56.6 (24.3)	52.9 (24.3)
IGF-II, ng/ml	421.4 (98.1)	428.5 (103.7)	424.8 (100.8)
FM/L^3 at Birth, kg/m^3	2.7 (1.2)	3.2 (1.2)	2.9 (1.2)
FM/L^3 at 2m, kg/m^3	5.9 (1.5)	6.2 (1.4)	6 (1.5)
FFM/L^2 at Birth, kg/m^2	11.8 (0.9)	11.5 (0.9)	11.7 (0.9)
FFM/L^2 at 2m, kg/m^2	12.8 (0.8)	12.1 (0.8)	12.5 (0.9)
Change in FM/L^3 z-score from birth to 2-months	0 (1.2)	0.1 (1.2)	0.05 (1.2)
Change in FFM/L^2 z-score from birth to 2-months	0.04 (1)	-0.02 (1.1)	0.01 (1)

4.5.4.1. Association of IGF-I and –II levels at birth with body composition at birth

Increased IGF-I concentrations were associated with higher FM/L^3 and FFM/L^2 z-scores at birth (R^2=-0.05, P<0.001 and R^2=0.02, P=0.016 respectively) (Figure 4.5.1). These associations were also seen separately in both sexes. IGF-II concentrations at birth were associated with FFM/L^2 z-score at birth (R^2=0.01, P=0.014) but not with FM/L^3 z-score at birth (R^2=0.002, P=0.3). When males and females were analysed separately, IGF-II concentration was only associated with FFM/L^2 z-scores at birth in females (R^2=0.02, P=0.034) and not males (R^2=0.005, P=0.2).

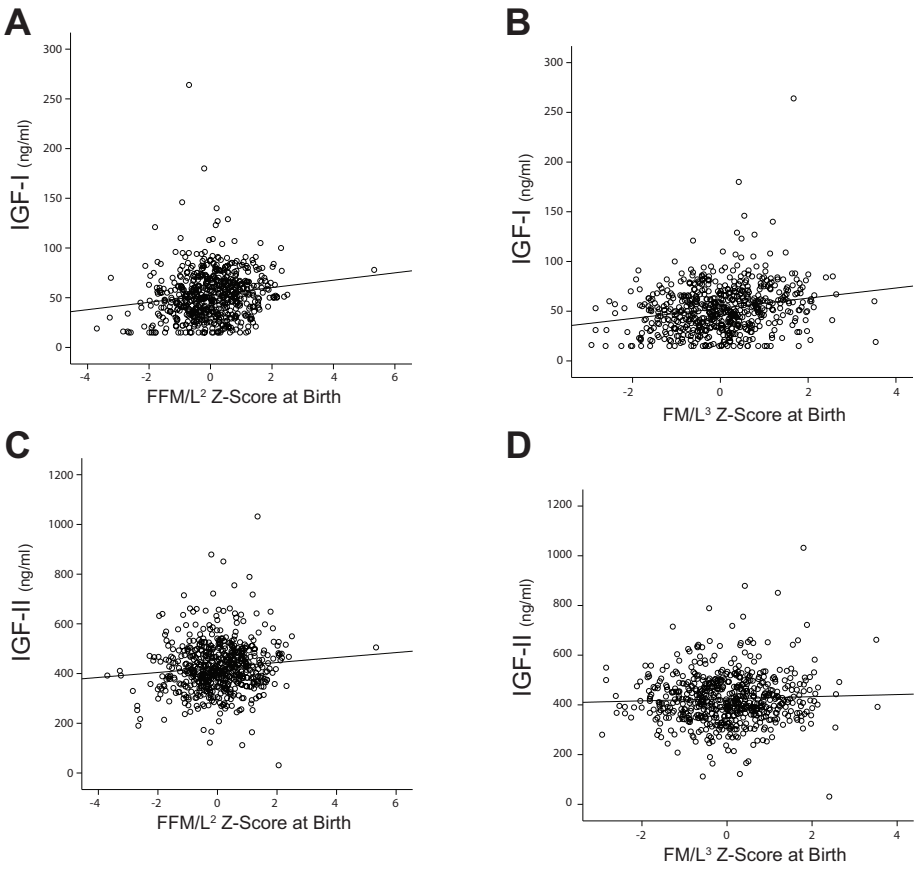

Figure 4.5.1: Scatter-plot and linear regression comparing IGF-I (A and B) and –II (C and D) concentrations with age- and sex-corrected FM/L^3 and FFM/L^2 z-scores at birth. The associations between IGF-I concentration and FM/L^3 and FFM/L^2 z-scores and the association between IGF-II concentration and FFM/L^2 z-score at birth were significant.

4.5.4.2. Association of IGF-I and –II at birth with body composition trajectory over the first 2 months

FM/L^3 and FFM/L^2 z-scores at two months were not associated with IGF-I (R^2=0.001, P=0.4 and R^2<0.01, P=0.8 respectively) or IGF-II (R^2=0.002, P=0.3 and R^2<0.002, P=0.3 respectively) concentrations at birth (Figure 4.5.2). This indicates that body composition at two months was not predicted by IGF-I or –II concentrations at birth. Separate analysis in males and females showed that there was also no sex-specific association.

However, the change in FM/L^3 and FFM/L^2 z-score was associated with IGF-I concentrations at birth and the change in FM/L^3 was associated with IGF-II concentration at birth. Higher IGF-I and/or IGF-II concentrations were associated with a reduction in FM/L^3 z-score between birth and two months,

although the association was stronger for IGF-I than for IGF-II concentrations (R^2= 0.04, P<0.001, and R^2=0.007, P=0.04 respectively). When males and females were analysed separately, IGF-II concentrations at birth were predictive of changes in FM/L^3 in females (R^2=0.03, P=0.006) but not males (R^2<0.001, P=0.8) (Table 4.5.2).

IGF-I concentrations at birth were associated with changes in FFM/L^2 in males (R^2<0.036, P=0.001) but not females (R^2<0.007, P=0.17).

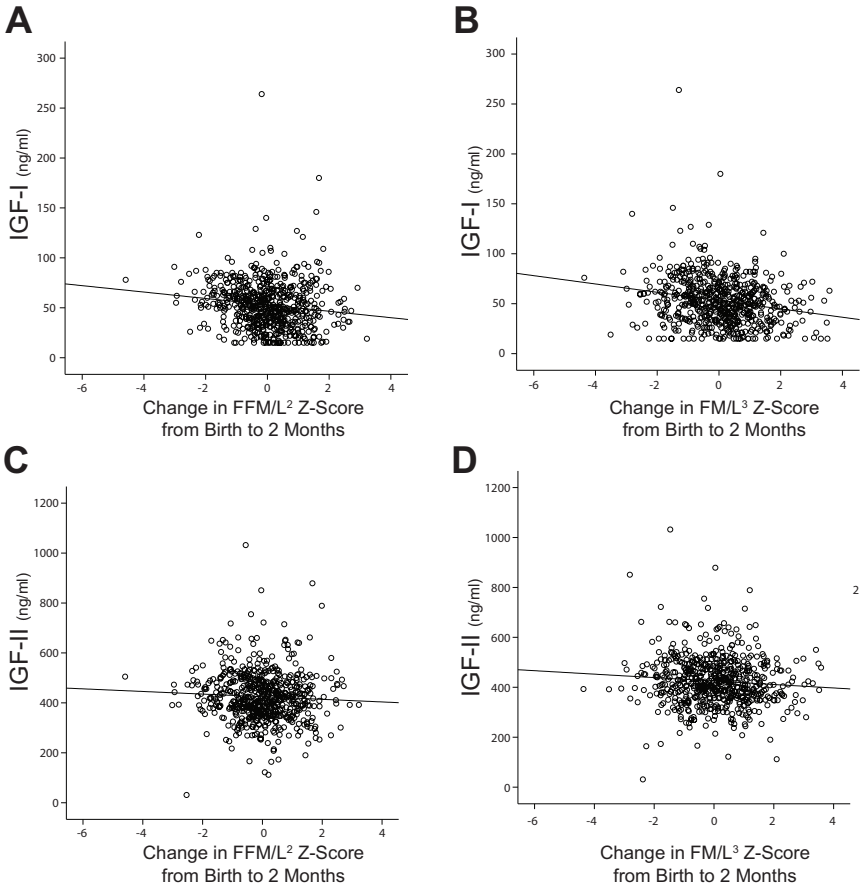

Figure 4.5.2: Scatter-plot and linear regression comparing IGF-I and –II concentrations at birth with age- and sex-corrected FM/L^3 and FFM/L^2 z-scores at 2 months.

Table 4.5.2: The relationship between sex- and gestational age-corrected FM/L^3 and FFM/L^2 z-scores at birth and years, with IGF-I and IGF-II concentrations at birth. For this analysis, IGF-I and –II are the dependent variables and FM/L^3 or FFM/L^2 measures are independent variables.

	IGF-I			IGF-II		
	R^2	Regression coefficient (SEM)	p	R^2	Regression coefficient (SEM)	p
Males						
FM/L^3 z-score at birth	0.048	5.04 (1.26)	<0.001	0	0.37 (5.3)	0.95
FM/L^3 z-score at two months	0.001	-0.56 (1.32)	0.67	0	-1.06 (5.42)	0.85
FFM/L^2 z-score at birth	0.025	3.85 (1.35)	0.005	0.005	7.24 (5.58)	0.2
FFM/L^2 z-score at two months	0.001	-0.8 (1.36)	0.56	0	0.07 (5.56)	0.99
Change in FM/L^3 z-score from birth to two months	0.04	-4.12 (1.09)	<0.001	0	-1.02 (4.56)	0.82
Change in FFM/L^2 z-score from birth to two months	0.036	-4.52 (1.32)	0.001	0.005	-6.96 (5.5)	0.21
Females						
FM/L^3 z-score at birth	0.047	5.26 (1.42)	<0.001	0.008	9.04 (6.19)	0.14
FM/L^3 z-score at two months	0.004	-1.52 (1.4)	0.28	0.012	-11.1 (6)	0.07
FFM/L^2 z-score at birth	0.02	3.35 (1.4)	0.016	0.016	12.67 (5.95)	0.034
FFM/L^2 z-score at two months	0.004	1.53 (1.45)	0.29	0.009	9.71 (6.19)	0.12
Change in FM/L^3 z-score from birth to two months	0.05	-4.48 (1.1)	<0.001	0.03	-13.49 (4.92)	0.006
Change in FFM/L^2 z-score from birth to two months	0.007	-1.8 (1.3)	0.17	0.001	-3.46 (5.62)	0.54
ALL						
FM/L^3 z-score at birth	0.05	5.1 (0.95)	<0.001	0.002	4.23 (4.03)	0.29
FM/L^3 z-score at two months	0.001	-0.87 (0.97)	0.37	0.003	-5.64 (4.02)	0.16
FFM/L^2 z-score at birth	0.024	3.7 (0.97)	<0.001	0.01	10 (4.1)	0.014
FFM/L^2 z-score at two months	0	0.3 (1)	0.76	0.002	4.62 (4.1)	0.27
Change in FM/L^3 z-score from birth to two months	0.04	-4.17 (0.79)	<0.001	0.007	-6.9 (3.35)	0.04
Change in FFM/L^2 z-score from birth to two months	0.019	-3.21 (0.94)	0.001	0.003	-5.25 (3.9)	0.18

4.5.5 DISCUSSION

This study has demonstrated the relationships between IGF-I and -II concentrations at birth and neonatal body composition in a large well-defined cohort. Higher IGF-I concentrations at birth are associated with increased adiposity and lean mass, while increased IGF-II concentrations are associated with increased lean mass in females only. IGF-I and –II concentrations at birth are not predictive of body composition parameters at two months, however they are predictive of rates of change in body composition during the first two months of life. Lower IGF-I concentrations at birth are strongly associated with an increase in adiposity in both sexes, but only an increase in lean mass in males. Low IGF-II concentrations at birth are associated with increased adiposity in females only.

The relationship between IGF-I concentration at birth and adiposity is not surprising. There is a strong association between IGF-I concentrations and birth weight[282, 349], as well as with nutritional status[196]. IGF-II is an important prenatal growth factor. Chorionic villi expression of *IGF2* mRNA is associated with birth weight[597] and paternally inherited *IGF2* mutations are associated with growth restriction[329]. Animal models also support this important role of IGF-II in regulating prenatal growth[598]. It is possible that IGF-II is more critical to growth prior to the third trimester[352, 353], and this may explain the absence of association between body composition and IGF-II concentrations at birth.

Although IGF-I and –II concentrations at birth are not directly associated with body composition parameters at two months, there is an association with trajectory of accumulation of adiposity and lean mass. Significant changes occur in body composition during the first three months of life, with a two-fold increase in FM corrected for length. Lean mass remains relatively stable during this time[270]. The inverse relationship between IGF-I concentration at birth and the change in adiposity may simply reflect the early “catch up” accumulation of body fat in infants with relative intrauterine growth restriction at birth. The same inverse association was seen between lean mass and IGF-I concentration at birth,

indicating that this "catch-up" is not limited to adiposity but also occurs in lean mass.

The consequences of this relationship may extend to the interplay between intrauterine nutritional status, the GH / IGF axis and the postnatal fetal programming of adult disease. Pregnancies complicated by limited access to nutrition are associated with increased IGF-I concentrations in adulthood, and it is hypothesised that chronic IGF suppression *in utero* results in an irreversible "overshoot" of this axis in later life[599]. Although we do not have IGF-I or –II concentrations measured in these infants at two months, it is possible that the rebound effect of chronic intrauterine suppression of these growth factors is demonstrated this early with rapid accumulation of adiposity, and possibly lean mass. Children who have rapid early weight gain have increased BMI and insulin resistance as early as eight years of age[600], and SGA children with rapid early weight gain have persistent increases in adiposity when compared with similar weight control children who were not born SGA[601]. Although the mechanisms for these persistent metabolic complications are unknown, it is possible that low IGF-I concentration at birth is a biomarker for future risk.

We have shown sex-specific associations between IGFs and early trajectories in body composition. Over the first two months of life, increases in adiposity are inversely associated with IGF-II concentrations at birth in females but not males whereas, conversely, increases in lean mass over the first two months were inversely associated with IGF-I concentrations at birth in males and not females. At birth, females have more body fat than males despite lower birth weight[269] and there are gender specific differences in visfatin and high molecular weight adiponectin concentrations at birth in SGA infants, reflecting gender differences in body fat distribution[267]. There are also differences in the GH / IGF axis at birth with females having higher IGF-I concentrations, and GH secretion may also be more sensitive to negative feedback from IGF-I concentrations in females[254]. Further complicating interpreting our data is the onset of mini-puberty within the observed two-month window in many infants[602, 603], and this

increased exposure to androgens may confound the changes in lean mass that we have described.

Strengths of this study include the large, well-defined, relatively homogenous study population. All children were term, healthy and Irish, making confounding factors unlikely to contribute to these findings. The large number of children with available body composition measurement is also unique for investigating these relationships. IGF-I and –II measurement by standard RIAs can be subject to assay interference by IGF binding proteins. However, LCMS was used in this study and this technique is not subject to this interference from binding proteins[238, 241]. A weakness of the study is the lack of available IGF-I and –II measurements at two months, to further investigate the hypothesis that IGF-I and -II may be useful biomarkers of metabolic risk during catch-up growth. Also, this study only included term infants and it is not known if the association is similar for preterm infants. Data were not available for the entire birth cohort, and it is not known if this introduced bias to our analysis.

4.5.6 Chapter Conclusion

IGF-I concentrations at birth are associated with adiposity and lean mass at birth and the trajectory of FM and FFM accumulation over the first two months. IGF measurement may have a role in evaluating body composition at birth and determining risk of rapid early changes in body composition and future metabolic risk. Sex differences in the association of IGF-I and -II concentrations at birth with body composition trajectories over the first two months of life exist and require further investigation.

SECTION 5

CONCLUSION OF THESIS

5.1: OVERVIEW

5.1.1 MAIN FINDINGS OF THIS RESEARCH

In this thesis, I have explored a number of new approaches to improving the diagnostic evaluation of children with short stature, and investigating disorders of the GH/IGF-I axis. I have also described reference data for body composition in infancy and explored how this relates to IGF-I and –II concentrations at birth. Figures 5.1 and 5.2 provide a pictorial overview of the components of the clinical evaluation that I have studied, and demonstrates how my work contributes to the current clinical approach to the child with short stature or poor growth. The main findings of each section of my thesis follow.

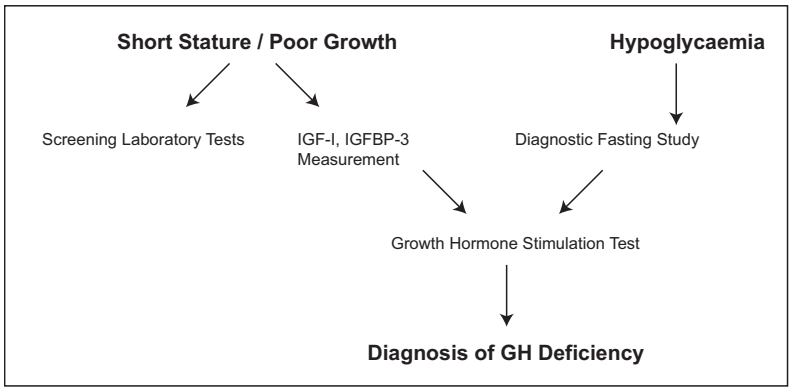

Figure 5.1: The current clinical approach to disorders of the GH/IGF-I axis

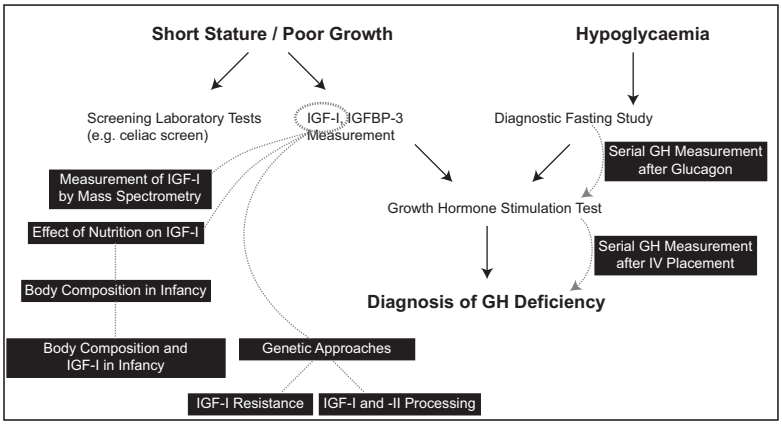

Figure 5.2: The contributions of this thesis to the established literature supporting the current clinical approach to disorders of the GH/IGF-I axis

5.1.1.1 Section 1: The Diagnosis of GHD in Childhood

Here, I explored modifications to the GHST in an effort to improve the specificity of the evaluation of children with suspected GHD. In children undergoing GHST, serial GH measurement after IVP and prior to administering a GH secretagogue in the context of a GHST is worthwhile. This will more than double the number of children who are considered GH sufficient based on this test[206]. Where infants are being evaluated for unexplained hypoglycemia with a diagnostic fasting study, modifying the study to include serial GH measurements after glucagon administration will increase the specificity of the test for GHD from 10% to 65%[604, 605].

5.1.1.2 Section 2: Mass Spectrometry and IGF-I Measurement

In Section 2, I have described that the reported normal concentrations of IGF-I in the first 18 months of life vary considerably in all reported studies that describe this measurement in healthy infants[124]. I considered assay interference as a possible explanation for this discrepancy, and then described reference data for IGF-I and -II concentrations at birth using a new LCMS assay. Using this assay, IGF-I and –II concentrations at birth were associated with weight, length, and OFC at birth. Low IGF-I concentrations at birth were associated with accelerated increases in weight and OFC z-scores over the first two months.

5.1.1.3 Section 3: Genetic Approaches to Disorders of the GH/IGF-I Axis

In Section 3, I have shown that despite GRP94's putative mechanism for affecting IGF-I production and supporting lab-based data, heterozygous mutations that affect protein function do not correlate with reduced IGF-I concentrations or short stature in the normal population[606]. I have also demonstrated that it is possible to use the EHR to identify children with a clinical phenotype consistent with IGF-I resistance, and used this approach to identify one child with a possibly pathogenic mutation of the IGF-IR.

5.1.1.4 Section 4: Nutrition and the GH/IGF-I Axis

In Section 4, I have described normal body composition at birth[269] and within the first two months of life[270]. Despite increased weight at birth, males have less fat than females[269]. When corrected for length, adiposity doubles while lean mass remains stable during the first two months of life[270]. Higher IGF-I concentrations at birth are associated with increased adiposity and lean mass. IGF-I and –II concentrations at birth are associated with rates of change in body composition in the first two months of life.

5.1.2 Summary of Impact on my Clinical Care

This research has already influenced my clinical practice, and my studies have changed the way in which I interpret the results of many of the diagnostic tests used in children with unexplained short stature.

Clinically, I now frequently request random GH concentrations when measuring IGF-I levels in children with short stature for whom I am considering GHD as a possible diagnosis. While low or undetectable GH concentrations do not necessarily warrant further investigation, phlebotomy can be a stimulus for GH secretion[206] and, occasionally, reassuringly high GH concentrations can be seen after phlebotomy in GH sufficient children. Similarly, poor nutrition can cause GH resistance[196] and may be associated with a random GH level being high in a child with a low IGF-I concentrations. Of course, other causes of GH resistance in addition to undernutrition should also be considered in this setting. This approach may help to redirect the diagnostic evaluation away from GHD and prevent unnecessary GHST and/or GH treatment.

In addition to changes to my approach to testing for GH reserve, I also have also generated data to support interpreting IGF-I concentrations in infancy. This research has prompted me to receive a number of "second opinion" clinical referrals. One of these cases, in particular, highlights how this research has influenced my decision-making. I recently received a second-opinion referral of an 11-month old patient. He was born at normal birth weight following an

uncomplicated pregnancy and delivery, and had been well until five months of age. Over the following three months, for unexplained reasons, he did not gain weight and started to show some motor developmental delay with central hypotonia. Following dietetic and physiotherapy support, his nutritional status improved and his weight and development now resembled that of a normal 11-month old.

His pediatric endocrinologist measured IGF-I concentrations as part of his otherwise normal diagnostic laboratory evaluation. His IGF-I concentration was measured by Quest's LCMS assay and was reported as undetectable. His IGFBP-3 concentration was within the normal range. His endocrinologist wanted to perform an MRI of his pituitary to rule out pituitary malformations, and also requested adrenal stimulation testing prior to proceeding to pituitary imaging under general anesthesia.

The work undertaken in this thesis led me to recommend an alternative approach on this occasion. Firstly, the reference data currently used by Quest in reporting IGF-I concentrations is based on samples from 43 children under one year of age and using a chemoluminescent immunoassay rather than LCMS[318]. I have shown wide variability in the reported IGF-I levels in children under one year of age[124]. I also know that many normal infants at birth have undetectable IGF-I by the LCMS assay (Chapter 2.4). Poor nutrition will also increase the likelihood of undetectable IGF-I concentrations even further[196]. This child's linear growth was now reassuring, developmental delay had resolved, and nutritional status was improving. Thus, the only indication for imaging was now an undetectable IGF-I concentration. He did not have hypoglycemia[605] or micropenis. I repeated the IGF-I measurement following a further two months of improved nutrition. He remained well during this time, continued to have normal growth, and his IGF-I measurement was improved at 29 ng/ml (Z-Score -1). He did not undergo general anaesthesia and MRI pituitary.

5.1.3 Summary of Contribution to the Medical Literature and Possible Future Directions

The studies included in this thesis have already led to ten peer-reviewed publications (Appendices A, B, D, F, G, H, J, K, L, N)[62, 124, 196, 206, 211, 269, 270, 604, 606], one book chapter (Appendix C)[607] and five presentations at international meetings (Appendices E, I, M, O, and P). An additional two manuscripts were submitted for publication at the time of thesis completion (Chapters 2.4 and 4.5).

5.1.3.1 Section 1

The limitations of GHST in diagnosing GHD are well established, and the recent guidelines for GH treatment in GHD strongly recommend against using the GHST as the sole diagnostic criterion of GHD[72]. These guidelines also refer to the difficulty in differentiating children with partial GHD from children with normal GH secretion, and recommend sex-steroid priming in select patients to improve the diagnostic yield. In Section 1 of this thesis, I have described two studies that may further improve the diagnostic yield of the GHST. IVP may be utilised as an additional stimulus to further improve the diagnostic utility of this test.

Prior to my study of serial GH measurement after glucagon administration in the context of a fasting study, the two most helpful studies in this area confirmed that GH concentrations during spontaneous hypoglycaemia is poorly specific for GHD[12] and spontaneous hypoglycaemia is associated with a blunted GH response when compared to insulin induced hypoglycaemia[185]. I have developed and studied a way to integrate a GH test into the diagnostic evaluation of children with unexplained hypoglycaemia. This can improve the specificity of the fasting study test for GHD. This approach has been adopted as routine care at CHOP, the largest referral centre for children with hypoglycaemia in North America. Potential future modifications for this approach might include using intramuscular, rather than intravenous, glucagon.

5.1.3.2 Sections 2 and 4

In Section 2, I have described assay variability in IGF measurement and explored the utility of a new LCMS assay for IGF-I and –II. Variation between commonly used immunoassays is a significant issue[260]. Although this LCMS assay has already been shown to be unaffected by IGFBP interference[238, 241], we have not demonstrated that LCMS provides a clinically relevant improvement in the diagnostic utility of the test. Future studies in this area will need to directly compare IGF measurement between this assay and commonly available immunoassays.

The observation that low IGF-I concentrations at birth are associated with weight gain (Chapter 2.4) and increases in length adjusted-FM and –FFM over the subsequent two months is potentially significant. Rapid weight gain in early infancy can be associated with cardiovascular morbidity[586, 608-610], premature adrenarche[611, 612], polycystic ovarian syndrome[613], visceral fat excess[609] and obesity[601]. This appears to be particularly concerning in children with prenatal growth restriction. Although the mechanisms for these associations are not clear, it is possible that the future management of children at risk of developing these complications may include monitoring the rates of change in body composition.

My reference data may provide a guide in determining whether an infant's FM/L^3 or FFM/L^2 is within the normal range and this may be a parameter to follow. Similarly, low IGF-I concentration at birth may identify children at risk of excessive 'catch-up' growth and identify infants for closer observation. IGF-I levels have been suggested as a marker of the nutritional state, so it is also possible that serial IGF-I measurement in infancy may have a role in monitoring weight gain and evolving metabolic risk in infancy. In order to investigate this further, there may be utility in studying serial IGF-I and –II concentrations during the first year of life, and determining if these are useful markers for changes in body composition during infancy.

The gender-specific relationships between IGF-I and –II and body composition trajectories add to the data on differences between metabolic parameters in males and females at birth[267], and may be a source of further study. We did not find a strong association for IGF-II concentrations at birth with body composition or growth, and the role of IGF-II in infancy warrants further study.

5.1.3.3 Section 3

As the cost of genetic testing reduces and this diagnostic modality becomes more accessible, genetic studies may be performed earlier in the evaluation of children with short stature in the future. There are known monogenic causes of short stature[358], but previously undescribed variants provide a diagnostic dilemma when found. A mutation in *GRP94*, for example, had a possible mechanism for causing short stature, and supporting laboratory data, but did not impact upon adult height when studied on a population level. Future studies into the P300L variant may explore effects on glucose homeostasis or immune function. Our research in novel mechanisms for IGF-I resistance will continue through *in vitro* studies of the mutation that we have identified. We have also developed a mechanism for collaboration in genetic studies of growth between three of the largest children's hospitals in the US, and may explore other rare growth phenotypes in the future.

5.1.4 MY FUTURE DIRECTIONS

This thesis has laid the groundwork for current research studies of mine that are in various stages of development. I currently have a "Junior Investigator Pilot Grant" award to study the potential role of body composition, muscle strength, bone density and cardiovascular parameters in predicting the clinical response to GH treatment in paediatric patients (5.1.4.1). Furthermore, I am a member of active research collaboration between CHOP and CCHMC looking to identify novel genetic aetiologies of short stature in children (5.1.4.2).

The hypercalcaemia and vitamin D intoxication study mentioned in Chapter 2.3 also opened up another avenue of research for me (5.1.4.3). Specifically, I am now investigating a potential role for rifampicin in the management of hypercalcaemia in children and adults with biallelic inactivating *CYP24A1* mutations[614]. I have also described a novel clinical phenotype of hypercalcaemia in children on a ketogenic diet[615].

5.1.4.1 Growth hormone sensitive short stature in childhood; A novel multisystem approach to diagnosis and characterising the effects of treatment

GH mediates many non-growth related effects that guide treatment in adult GHD, but these are largely ignored in the diagnosis of pediatric GHD and in the monitoring of treatment efficacy[616]. The multisystem phenotype of adult GHD is well described. This includes reductions in lean body mass, exercise tolerance, fractional shortening of cardiac myocytes and high-density lipoprotein (HDL) as well as increases in fat mass, central adiposity, intima media wall thickness, triglycerides and low-density lipoprotein[617]. The effects of GH treatment on each of these systems may be dose-dependent[618], but whether or not adverse effects of partial deficiency on each of these systems correlates with severity of deficiency in children is not known. Given the poor sensitivity and specificity of the GHST for GHD (Section 1), these may represent additional tools for the diagnosis of GHD.

The primary objective of this funded study is to utilise many of the known physiological effects of GH to determine if these could be used in combination to improve the identification of children who would respond to GH treatment with a significant increase in linear growth. Secondary objectives are to determine if 1) early changes in these parameters will predict growth response in the first year of treatment and 2) GH treatment results in favorable effects on cardiometabolic risk, bone density, body composition, cardiac function and vascular endothelial function in pediatric patients. This successful grant application is shown in

Appendix Q. At the time of thesis submission, three subjects have been recruited to this study.

5.1.4.2 Identifying and studying the novel variants in the GH/IGF-I axis

We plan to build on our established genetics of growth collaboration between CHOP, BCH and CCHMC. We have developed processes for identifying children with rare growth phenotypes at CHOP through automated searches of the EHR. Similar processes are now in place at CCHMC and BCH, and a mechanism for sharing data has been established through a research collaborative agreement and parallel IRB approved research protocols. The development of this infrastructure has overcome a significant barrier to prior collaborative growth research.

We have now applied to the National Institute for Health for funding to continue this work. This R01 grant application was under review at the time of submission of this thesis (Primary Investigator: Dr Andrew Dauber).

5.1.4.3 Rifampicin in patients with homozygous *CYP24A1* mutations

My study of unexplained elevated $1,25(OH)_2D$ concentrations in vitamin D intoxication (Chapter 2.3)[211] was the start of my research path in calcium metabolism under the mentorship of Professor Michael Levine.

We have published a case series describing a novel hypercalcemia phenotype in patients on the ketogenic diet[615], which was the first study to describe this association. We have also published a novel approach to a rare but untreatable form of hypercalcaemia, namely homogenous inactivating mutations of *CYP24A1*. Incidentally, our collaborator, Dr Andrew Dauber was one of the first to describe this rare condition[619].

Dr Levine and I have described the potential of rifampicin to treat hypercalcaemia in these patients[614]. The National Institute for Health has funded

an R01 grant to prospectively study this therapeutic approach (Principal Investigator: Michael Levine) and this began in July 2017.

5.1.5 Personal Reflection

This has been a journey of personal growth, failures and successes. All of the preceding chapters and studies describe completed projects, most of which have made it all of the way to peer-reviewed publication. However, each success has been built on a foundation of numerous failures. I will summarise the most significant of these disappointments before reflecting on my personal growth.

5.1.5.1 Genetic markers of Growth Response to GH treatment

In the early stages of this thesis, an opportunity arose to collaborate with Dr Andrew Dauber, the Director of the Growth Center at CCHMC. He had performed whole exome sequencing on a large number of patients who had GHD and were treated with GH. The Center for Applied Genomics at CHOP has processed over 100,000 genomes in pediatric patients with detailed clinical phenotypes. My aim was to identify children from this database who had been treated with GH, and to combine our data with Dr Dauber's cohort. This may have been an important step towards identifying genetic markers of GH responsiveness.

However, after months of searching this database, it became apparent that there were not enough subjects recruited to the CHOP genetics study who had been treated with GH. Furthermore the clinical data available on the small number of patients were not sufficiently detailed to contribute to Dr Dauber's well-characterised cohort. This collaboration was abandoned, but this led to further work with Dr Dauber (Chapter 3.3 and 5.1.4.2).

5.1.5.2 Growth response to GH treatment in type 1 diabetes

Poor glycemic control in children with type 1 diabetes mellitus (T1D) attenuates peak height velocity and may delay the onset of puberty[620]. Height at onset of

diabetes, glycemic control and duration of diabetes influence final adult height[621, 622]. Thus, children with T1D are at an increased risk of impaired growth and this may lead to an evaluation for possible GHD. When compared with controls, children with T1D have lower IGF-I concentrations[623, 624]. This is most prominent in children without residual beta cell function and with poor glycaemic control[624]. In this context, it is possible that loss of negative feedback of IGF-I on the anterior pituitary results in increased GH production, which may contribute to increased insulin resistance and the cycle may continue with increased hyperglycemia.[625]

In children with T1D, there is the additional complication of the effect of GH treatment on glycaemic control. In a German study including 2-year follow up of 21 children with T1D treated with GH, there was an increase in median haemoglobin A1c (7.3 to 8.1%) and total daily insulin dose (0.7 to 1.1 u/kg/day). Height SDS did not change significantly during those two years of treatment (-2.3 to -2).

With this in mind, I hypothesised that a large number of children with T1D were being misdiagnosed as having GHD and receiving GH treatment. I also thought that a) it is likely that the growth response to treatment was poor as most of these children don't have GHD but do have GH resistance and b) GH treatment has additional complications in these patients due to the negative effect on glycaemic control. In order to test this hypothesis, I received funding from the Diabetes Center at CHOP to perform a retrospective review of the EHR to identify children with T1D treated with GH. The aim of the study was to determine if children with T1D who meet clinical criteria of GHD have a growth response to GH treatment that is similar to children without GHD.

Preliminary search of the EHR indicated that there were more than 100 children with diabetes who were treated with GH and this fuelled my enthusiasm for this project. However, once all of the data were extracted from the EHR, it became apparent that the EHR had miscoded many children with diabetes insipidus as

having T1D. Only nine children with T1D were treated with GH and, of those, only four had complete data available for review. I decided that this was not sufficient to test the hypothesis, and the study was abandoned.

5.1.5.3 GRP94

The original title for this thesis was "Altered function in Glucose Regulated Protein 94: A novel mechanism of intra-uterine growth restriction and primary IGF-I deficiency". However, when I started to learn the laboratory skills to investigate this further, Dr Marzec had already completed many studies describing the pathogenicity of the most common polymorphism (P300L, Chapter 3.2). I had begun to work on describing the clinical phenotype, and noted that the laboratory findings did not result in a significant effect on growth or IGF-I production. There were a number of rarer genetic mutations identified in the *grp94* gene in these populations that could have been investigated further with *in vitro* and population studies. However, I considered it likely that further research on this particular gene would not result in significant clinically relevant data. I turned my attention to clinical research, which is my main research interest and redirected my PhD under the supervision of my mentorship team.

5.1.6 Personal Growth

Over the past three years, I have developed a number of important research skills and attained many of the necessary tools for a career in clinical research. For this, I am grateful to my mentorship team for their guidance, support and advice.

In order to gain the necessary research skills, I have attended courses at the University of Pennsylvania in biostatistics, data management and in reviewing medical literature. I have also attended courses at CHOP in good clinical practice for research trials and responsible conduct of research, as well as seminars hosted by the IRB to gain insight into the process of ethical review at CHOP.

Through direct mentorship, I have been guided in hypothesis development, data management, data analysis and manuscript preparation. My biggest challenge has been performing studies with clear aims and ensuring that the data were directly focused on the central aim of my study. I am especially grateful to Prof Grimberg and Dr Murray for helping me to improve in this area. The guidance that I have received in attaining the necessary research skills for success has been invaluable to me. I have already started to use these skills in mentoring more junior trainees as the senior author on their research[626, 627]. In addition to the generic research skills that I have learned from my mentors, I hope to have absorbed some of their patience, willingness to teach and accessibility.

I have also learned the importance of carving out my own research path and to follow directions that I believe to be both interesting and important. The most difficult part of this PhD was the decision to redirect my studies away from the research laboratory and towards clinical studies. Professor Argon had provided me with a lot of support in developing laboratory skills and writing grant applications. I am extremely grateful to him, both for providing me with this opportunity and, more importantly, for supporting my decision to redirect my research away from the laboratory. I believe that this decision has been responsible for the success that my research has brought and ensured that I continued to enjoy this experience.

The most important lesson learned in the past few years, however, is the value of strong and diverse mentorship and collaboration. Without the selfless support of all of the mentors listed in the acknowledgements section of this thesis, I would not have had the opportunity to undergo this personal development or to contribute to the field of growth research. The friendships that I have built in the course of completing this thesis will be invaluable as I progress through my career.

I also recognise that the sacrifices required for me to complete this thesis were not just made by me. My wife and children have also been important members of my team, and this work would not have been possible without their support.

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APPENDICES

Appendix A: Hawkes CP, O'Connell SM. Growth hormone use in Ireland – A national survey of practice. Irish Medical Journal. 2016:109

Appendix B: Hawkes CP, Mavinkurve M, Fallon M, Grimberg A, Cody DC. Serial GH measurement after intravenous catheter placement alone can detect levels above stimulation test thresholds in children. J Clin Endocrinol Metab. 2015;100(11):4357-63

Appendix C: Hawkes CP, Stanley CA. Pathophysiology of neonatal hypoglycemia. In: Polin RA, Fox WW, Abman SH, editors. Fetal and Neonatal Physiology: Expert Consult - Online and Print. 5 ed: Elsevier/Saunders; 2016. p. 1550-60

Appendix D: Hawkes CP, Grimberg A, Dzata VE, De Leon DD. Adding glucagon-stimulated GH testing to the diagnostic fast increases the detection of GH-sufficient children. Horm Res Paediatr. 2016;85(4):265-72

Appendix E: Hawkes CP, Grimberg A, Dzata VE, De Leon DD. Integrating growth hormone testing with hypoglycaemia investigation. American Pediatric Society / Society for Pediatric Research, May 2014. (Poster Presentation)

Appendix F: Hawkes CP, Grimberg A. Measuring growth hormone and insulin-like growth factor-I in infants: what is normal? Pediatr Endocrinol Rev. 2013;11(2):126-46

Appendix G: Hawkes CP, Schnellbacher S, Singh RJ, Levine MA. 25-hydroxyvitamin D can interfere with a common assay for 1,25-dihydroxyvitamin D in vitamin D intoxication. J Clin Endocrinol Metab. 2015;100(8):2883-9

Appendix H: Hawkes CP, Murray DM, Kenny LC, Kiely M, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Correlation of insulin-like growth factor-I and –II concentrations at birth measured by mass spectrometry and growth from birth to two months. Horm Res Paediatr. 2018 Jan. doi 10.1159/000486035 [Epub ahead of print].

Appendix I: Hawkes CP, Murray DM, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Measurement of IGF-I and –II concentrations at birth by mass spectrometry in a large birth cohort: correlation with anthropometry. Pediatric Endocrine Society, Washington DC. September 2017 (Poster Presentation)

Appendix J: Marzec M, Hawkes CP, Eletto D, Boyle S, Rosenfeld R, Hwa V, et al. A human variant of glucose-regulated protein 94 that inefficiently supports IGF production. Endocrinology. 2016;157(5):1914-28

Appendix K: Hawkes CP, Grimberg A. Insulin-like growth factor-I is a marker for the nutritional state. Pediatr Endocrinol Rev. 2015;13(2):465-77

Appendix L: Hawkes CP, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Gender- and gestational age-specific body fat percentage at birth. Pediatrics. 2011;128(3):E645-E51

Appendix M: Hawkes CP, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Body Composition at birth; normative values. American Pediatric Society / Society for Pediatric Research. Denver, May 2011. European Society for Paediatric Research, Newcastle, Sept 2011

Appendix N: Hawkes CP, Zemel BS, Kiely M, Irvine AD, Kenny LC, O'B Hourihane J, Murray DM. Body composition within the first 3 months: optimized correction for length and correlation with BMI at 2 years. Horm Res Paediatr. 2016;86(3):178-187

Appendix O: Hawkes CP, Zemel BS, Kiely M, Irvine A, Kenny LC, O'B Hourihane J, Murray DM. Body composition in the first 2 months of life – optimized correction for length, reference data and correlation with obesity at 2 years. American Pediatric Society / Society for Pediatric Research. Baltimore, May 2016

Appendix P: Hawkes CP, Zemel BS, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life. Pediatric Endocrine Society, September 2017 (Poster Presentation)

Appendix Q: Junior Investigator Pilot Grant Application

***Appendix A**:*

Hawkes CP, O'Connell SM. Growth hormone use in Ireland – A national Survey of practice. Irish Medical Journal. 2016:109

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***Appendix B**:*

Hawkes CP, Mavinkurve M, Fallon M, Grimberg A, Cody DC. Serial GH Measurement After Intravenous Catheter Placement Alone Can Detect Levels Above Stimulation Test Thresholds in Children. J Clin Endocrinol Metab. 2015;100(11):4357-63.

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***Appendix C**:*

Hawkes CP, Stanley CA. Pathophysiology of Neonatal Hypoglycemia. In: Polin RA, Fox WW, Abman SH, editors. Fetal and Neonatal Physiology: Expert Consult - Online and Print. 5 ed: Elsevier/Saunders; 2016. p. 1550-60.

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***Appendix D**:*

Hawkes CP, Grimberg A, Dzata VE, De Leon DD. Adding Glucagon-Stimulated GH Testing to the Diagnostic Fast Increases the Detection of GH-Sufficient Children. Horm Res Paediatr. 2016;85(4):265-72.

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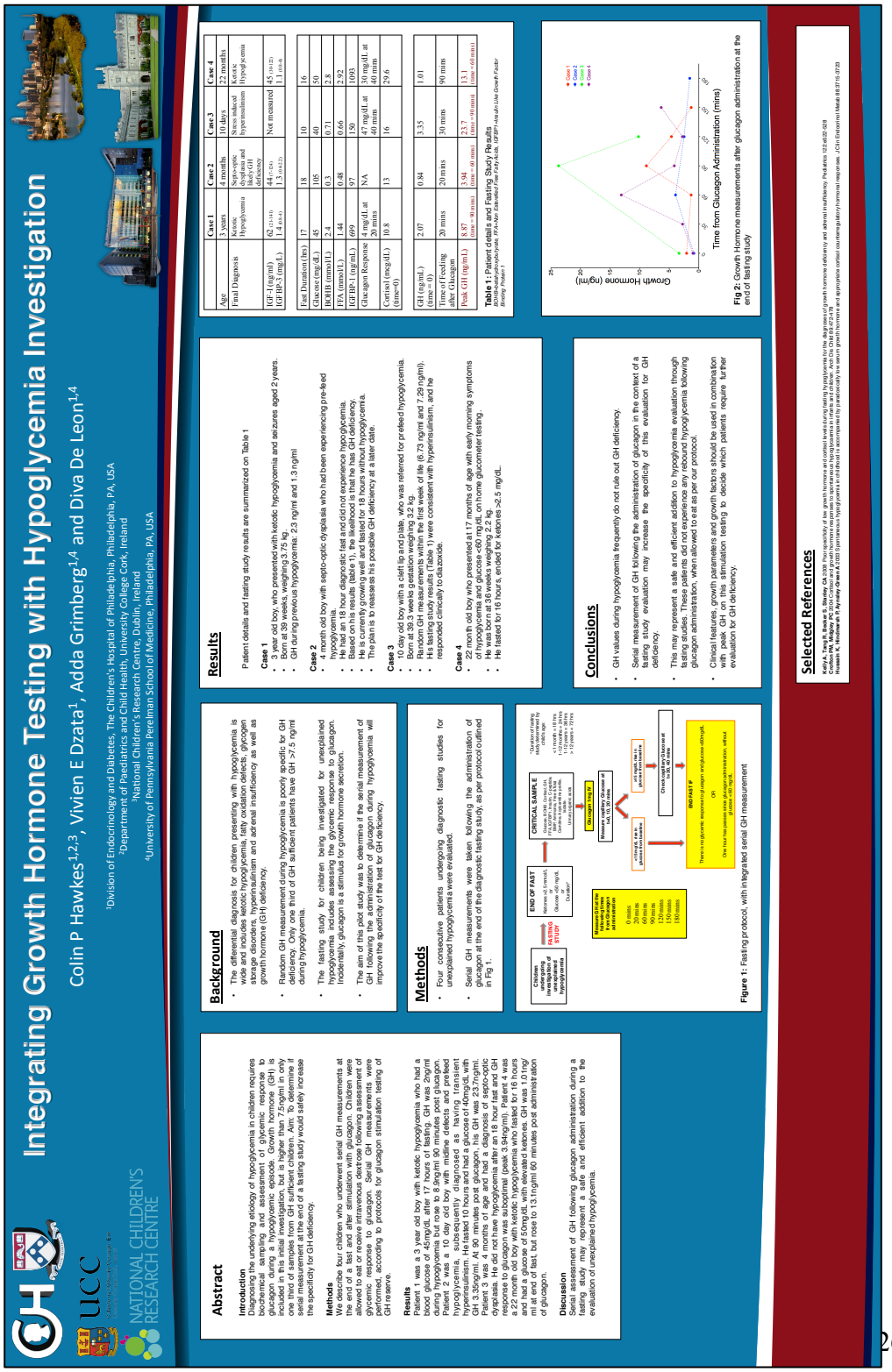
Appendix E:

Hawkes CP, Grimberg A, Dzata VE, De Leon DD. Integrating growth hormone testing with hypoglycaemia investigation. American Pediatric Society / Society for Pediatric Research, May 2014. (Poster Presentation)



***Appendix F**:*

Hawkes CP, Grimberg A. Measuring growth hormone and insulin-like growth factor-I in infants: what is normal? Pediatr Endocrinol Rev. 2013;11(2):126-46.

[Copyrighted publication not available for online archiving]

***Appendix G**:*

Hawkes CP, Schnellbacher S, Singh RJ, Levine MA. 25-Hydroxyvitamin D Can Interfere With a Common Assay for 1,25-Dihydroxyvitamin D in Vitamin D Intoxication. J Clin Endocrinol Metab. 2015;100(8):2883-9.

[Copyrighted publication not available for online archiving]

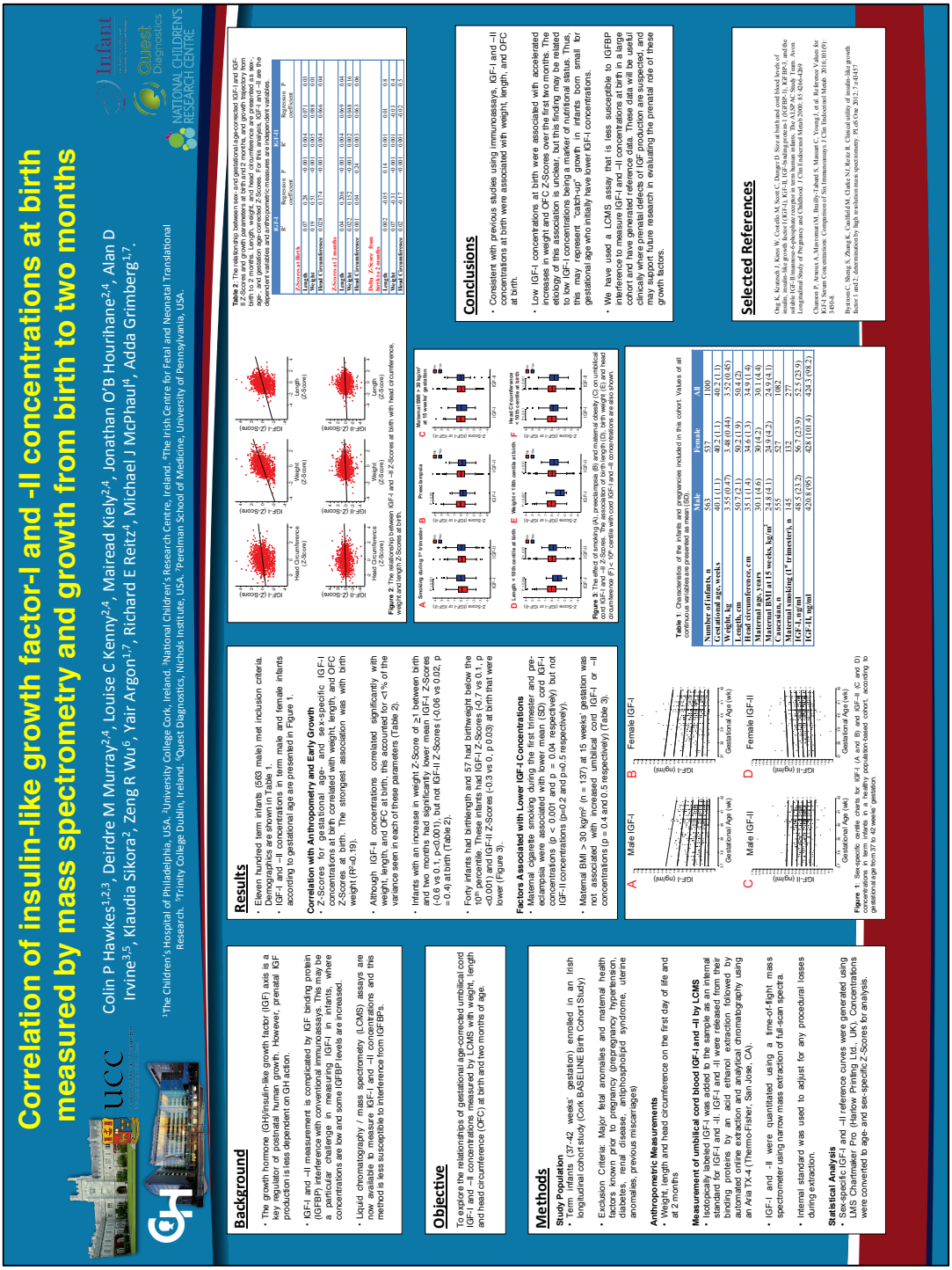
***Appendix H**:*

Hawkes CP, Murray DM, Kenny LC, Kiely M, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Correlation of insulin-like growth factor-I and –II concentrations at birth measured by mass spectrometry and growth from birth to two months. Horm Res Paediatr. 2018 Jan. doi 10.1159/000486035 [Epub ahead of print].

[Copyrighted publication not available for online archiving]

Appendix I:

Hawkes CP, Murray DM, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A. Measurement of IGF-I and –II concentrations at birth by mass spectrometry in a large birth cohort: correlation with anthropometry. Pediatric Endocrine Society, Washington DC. September 2017 (Poster Presentation)



***Appendix J**:*

Marzec M, Hawkes CP, Eletto D, Boyle S, Rosenfeld R, Hwa V, et al. A Human Variant of Glucose-Regulated Protein 94 That Inefficiently Supports IGF Production. Endocrinology. 2016;157(5):1914-28.

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***Appendix K**:*

Hawkes CP, Grimberg A. Insulin-like growth factor-I is a marker for the nutritional state. Pediatr Endocrinol Rev. 2015;13(2):465-77.

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***Appendix L**:*

Hawkes CP, O'B Hourihane J, Kenny LC, Irvine AD, Kiely M, Murray DM. Gender- and Gestational Age-Specific Body Fat Percentage at Birth. Pediatrics. 2011;128(3):E645-E51.

[Copyrighted publication not available for online archiving]

***Appendix M**:*

Hawkes CP, O'B Hourihane, J, Kenny LC, Irvine AD, Kiely M, Murray DM. Body Composition at birth; normative values. American Pediatric Society / Society for Pediatric Research. Denver, May 2011. European Society for Paediatric Research, Newcastle, Sept 2011.

Body Composition at Birth: The Effect of Sex and Gestational Age

[1,2]Hawkes CP, [1]O' B Hourihane J, [3]Kenny LC, [4]Irvine AD, [5]Kiely M, [1]Murray DM

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INTRODUCTION

In utero growth has an immediate and far-reaching influence on health

Assessment of neonatal adiposity is difficult, as birth weight centiles and skin fold thickness do not correlate well with body composition.

The PEAPOD™ (Life Measurement Inc, Concord, CA) uses air displacement plethysmography to measure body fat percentage (%BF) in infants (Fig 1). It has been shown to provide reliable and accurate measurements of %BF.

AIMS

1. **To describe %BF within the first 4 days of life, in infants born after 36 weeks' gestation**

2. **To detail the effect of infant sex and gestation on %BF**

METHODS

Data collected as part of an ongoing pregnancy/birth cohort study; the SCOPE/BASELINE study.

1. **SCOPE pregnancy study**: Recruits primiparous, low risk women at 15±1 weeks' gestation.
2. **BASELINE birth cohort**: The maternal-fetal dyads of the SCOPE study are recruited to the BASELINE birth cohort study

Infants have %BF measured within 4 days of life using the PEAPOD™.

Included in this study are infants between 36 and 41+6 weeks' gestation, born between March 2009 and October 2010.

	36-37+6wk	38-39+6wk	40-41+6wk	Total	p-value[c]
Number	45	243	455	743	
Male (%)	23 (51.1)	139 (57.2)	228 (50.1)	390 (52.5)	0.199
Mean Gestational Age in weeks (SD)	37.2 (0.6)	39.1 (0.5)	40.8 (0.5)	40.1 (1.2)	<0.001
Mean birthweight (g) (SD)	2955 (313)	3326 (422)	3643 (437)	3498 (470)	<0.001
Mean day of Peapod Measurement (SD)	2.1 (1)	1.9 (0.9)	1.8 (0.9)	1.9 (0.9)	0.064
Caucasian Ethnicity	44 (97.8)	240 (98.8)	447 (98.2)	731 (98.4)	0.826
Mean maternal age	29.8 (4.9)	29.7 (4.6)	29.7 (4.3)	29.7 (4.5)	0.999
Maternal University Degree or Higher (%)	15 (33.3)	102 (42)	222 (48.8)	339 (45.6)	0.053
Smoked in Pregnancy (%)	14 (31.1)	65 (26.7)	125 (27.5)	204 (27.5)	0.835
Mean Maternal BMI at 16 weeks' gestation (SD)	23.6 (4.2)	24.6 (4.1)	24.9 (4.1)	24.7 (4.2)	0.124
Socioeconomic Status[a](%)					
1	2 (4.4)	10 (4.1)	34 (7.5)	46 (6.2)	0.897
2	7 (15.6)	41 (16.9)	60 (13.2)	108 (14.5)	
3	18 (40)	91 (37.4)	176 (38.7)	285 (38.4)	
4	3 (6.7)	17 (7)	37 (8.1)	57 (7.7)	
5	4 (8.9)	42 (17.3)	69 (15.2)	115 (15.5)	
6	11 (24.4)	42 (17.3)	79 (17.4)	132 (17.8)	

Table 1: Demographic Data of the Study Population, categorised by Gestational Age
[a]New Zealand Socioeconomic Index [b]One –way ANOVA

Fig 1: Body Composition measurement using the PEAPOD™

	Male	Female	TOTAL	P-value[b]
36-37+6 weeks gestation	**23**	**22**	**45**	
Birthweight g (SD)	3099 (340)	2898 (277)	2955 (313)	0.24
Body Fat % (SD)*	8.8 (3.2)	8.9 (3.8)	8.9 (3.5)	0.968
Fat Free Mass % (SD)*	91.2 (3.2)	91.1 (3.8)	91.1 (3.5)	0.968
Surface Area cm^2 (SD)*	2049 (148)	2010 (112)	2030 (132)	0.317
Length (cm)	48.2 (2.5)	48.2 (1.7)	48.2 (2.1)	0.973
Abdominal Circumference (cm)	32.2 (1.9)	31.3 (1.4)	31.7 (1.7)	0.085
Midarm Circumference (cm)	9.8 (0.7)	9.7 (0.8)	9.7 (0.8)	0.681
38-39+6 weeks gestation	**139**	**104**	**243**	
Birthweight	3362 (436)	3279 (399)	3326 (422)	0.13
Body Fat % (SD)*	9.8 (3.9)	11.1 (3.9)	10.3 (4)	0.012
Fat Free Mass % (SD)*	90.2 (3.9)	88.9 (3.9)	89.7 (4)	0.012
Surface Area cm^2 (SD)*	2203 (172)	2159 (162)	2184 (169)	0.043
Length (cm)	49.8 (1.9)	49.1 (1.8)	49.5 (1.9)	0.004
Abdominal Circumference (cm)	33 (2)	32.9 (2)	33 (2)	0.806
Midarm Circumference (cm)	10.4 (1)	10.2 (0.9)	10.3 (1)	0.12
40-41+6 weeks gestation	**228**	**227**	**455**	
Birthweight	3687 (431)	3598 (440)	3643 (437)	0.029
Body Fat % (SD)*	10 (3.9)	12.5 (4.4)	11.2 (4.3)	<0.001
Fat Free Mass % (SD)*	90 (3.9)	87.5 (4.4)	88.8 (4.3)	<0.001
Surface Area cm^2 (SD)*	2335 (160)	2290 (164)	2312 (164)	0.003
Length (cm)	51.2 (1.8)	50.5 (1.7)	50.9 (1.8)	<0.001
Abdominal Circumference (cm)	33.9 (1.9)	33.7 (2)	33.8 (2)	0.526
Midarm Circumference (cm)	10.8 (1)	10.8 (1)	10.8 (1)	0.397
Total cohort	**390**	**351**	**743**	
Birthweight	3531 (472)	3460 (466)	3498 (470)	0.04
Body Fat % (SD)*	9.8 (3.9)	11.9 (4.3)	10.8 (4.2)	<0.001
Fat Free Mass % (SD)*	90.2 (3.9)	88.1 (4.3)	89.2 (4.2)	<0.001
Surface Area cm^2 (SD)*	2271 (184)	2234 (181)	2254 (183)	0.006
Length (cm)	50.5 (2)	50 (1.9)	50.3 (2)	<0.001
Abdominal Circumference (cm)	33.4 (2)	33.3 (2.1)	33.4 (2)	0.503
Midarm Circumference (cm)	10.6 (1)	10.6 (1)	10.6 (1)	0.911

Table 2:Male and Female Measurements at Different Gestational Ages
*Independent Sample t-test [b]ANOVA
*Denotes variables measured by PEAPOD

Centile	36-37+6 weeks			38-39+6 weeks			40-41+6 weeks		
	Male	Female	All	Male	Female	All	Male	Female	All
97.5[th]	14.9	17.5	17.1	19	18.2	18.4	18.2	22.1	19.8
95[th]	14.5	16.9	14.4	17.1	17.7	17.5	16.2	19.2	18.3
90[th]	13	13.1	12.9	14.5	16.3	15.5	15	17.9	16.7
75[th]	11.9	12	11.9	12.2	14.1	13	12.7	15.4	14.2
50[th]	9.2	8.9	9.2	9.6	11	10.3	9.9	12.5	10.9
25[th]	6	5.7	5.9	7.2	7.9	7.5	6.9	9.4	8.1
10[th]	4.6	4	4.4	4.7	6.2	5.1	4.9	7.2	5.8
5[th]	3.4	1.8	3.3	3.2	4.7	3.4	3.4	5.6	4.4
2.5[th]	3.1	1.4	1.7	2.4	2.9	2.6	2.8	4.7	3.2

Table 3: Centiles for %BF grouped by Gestational Age and Sex

RESULTS

793 recruited infants were delivered during study period. Of these 12 were outside of our gestational range, and 31 did not have body composition measured within 4 days. Demographic data is displayed on Table 1.

Mean(SD) body fat at birth was 10.8(4.2)%, but this varied significantly with sex and gestational age (Table 2). In each gestational age category males had lower mean %BF than females, with the difference increasing with gestational age (Table 3). This decreased %BF occurred despite increased birth weight and fat free mass

On stepwise linear regressional analysis, gestational age remained a significant association (R=0.193, p<0.001) when corrected for maternal BMI at 16 weeks' gestation, socio-economic group, maternal age, and cigarette consumption. Maternal BMI at 16 weeks' gestation also was significantly and consistently associated with %BF at birth.

CONCLUSIONS

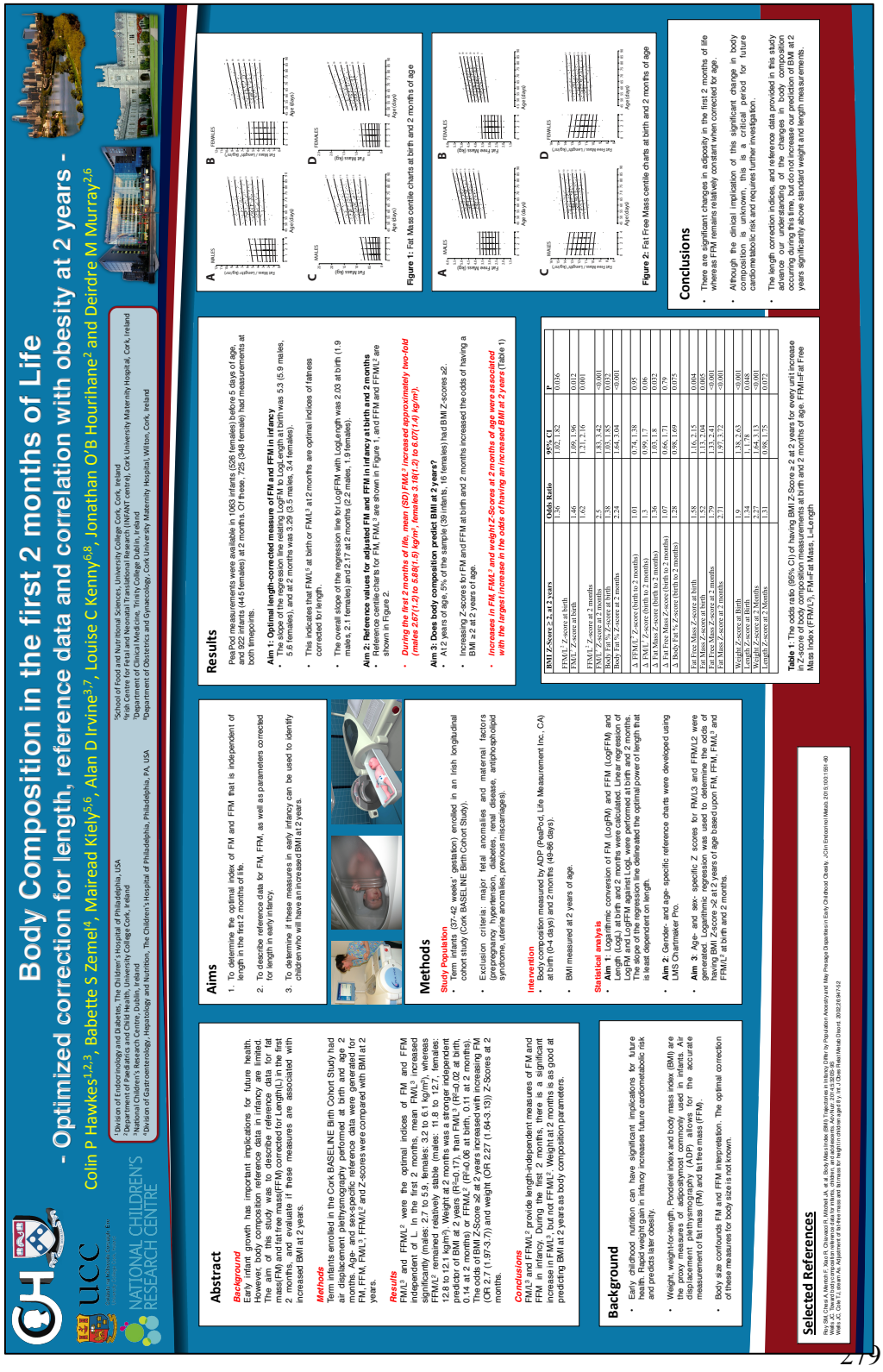
1. %BF increases with gestational age from 36 to 41+6 weeks' gestation.
2. Females have a greater %BF at birth than males, despite lower birthweight.

***Appendix N**:*

Hawkes CP, Zemel BS, Kiely M, Irvine AD, Kenny LC, O'B Hourihane J, Murray DM. Body Composition within the First 3 Months: Optimized Correction for Length and Correlation with BMI at 2 Years. Horm Res Paediatr. 2016;86(3):178-187.

Appendix O:

Hawkes CP, Zemel BS, Kiely M, Irvine A, Kenny LC, O'B Hourihane J, Murray DM. Body composition in the first 2 months of life – optimized correction for length, reference data and correlation with obesity at 2 years. American Pediatric Society / Society for Pediatric Research. Baltimore, May 2016



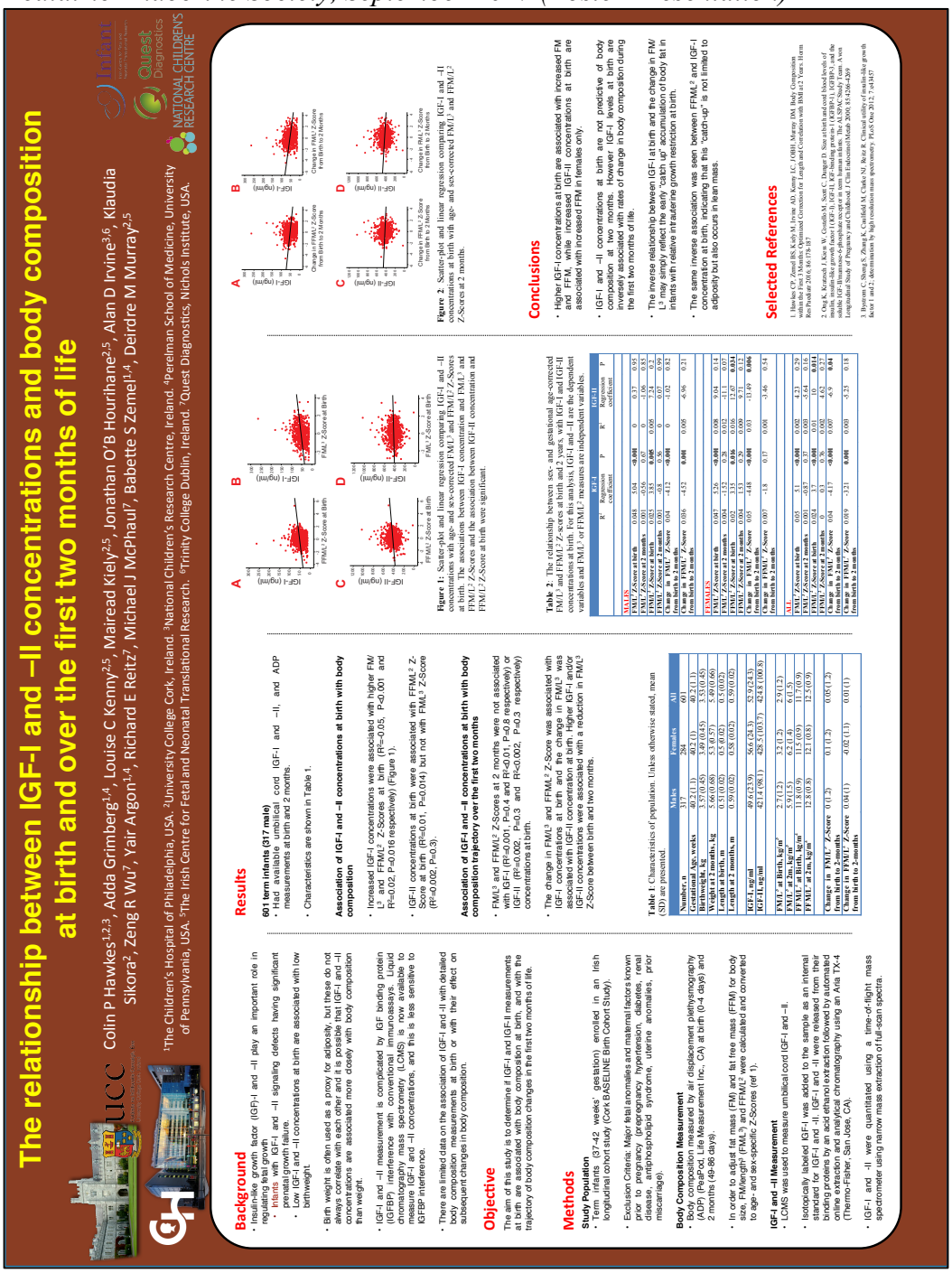
BMI Z-Score ≥ 2, at 2 years	Odds Ratio	95% CI	P
FFMI,^{2} Z-score at birth	1.36	1.02, 1.82	0.036
FMI,^{3} Z-score at birth	1.46	1.09, 1.96	0.012
FFMI,^{2} Z-score at 2 months	1.62	1.21, 2.16	0.001
FMI,^{3} Z-score at 2 months	2.5	1.83, 3.42	<0.001
Weight Z-score at 2 months	2.27	1.64, 3.04	<0.001
Body Fat % Z-score at birth	1.38	1.03, 1.85	0.032
Body Fat % Z-score at 2 months	2.54	1.84, 3.04	<0.001
Δ FFMI,^{2} Z-score (birth to 2 months)	1.01	0.74, 1.38	0.95
Δ FMI,^{3} Z-score (birth to 2 months)	1.3	0.99, 1.7	0.06
Δ Fat Mass Z-score (birth to 2 months)	1.56	1.03, 1.8	0.032
Δ Fat Free Mass Z-score (birth to 2 months)	1.07	0.66, 1.71	0.79
Δ Body Fat % Z-score (birth to 2 months)	1.28	0.98, 1.69	0.075
Fat Free Mass Z-score at birth	1.58	1.16, 2.15	0.004
Fat Mass Z-score at birth	1.52	1.13, 2.04	0.005
Fat Free Mass Z-score at 2 months	1.79	1.33, 2.41	<0.001
Fat Mass Z-score at 2 months	2.71	1.95, 3.72	<0.001
Weight Z-score at Birth	1.9	1.38, 2.63	<0.001
Length Z-score at Birth	1.24	1.01, 1.53	0.048
Weight Z-score at 2 Months	2.27	1.64, 3.13	<0.001
Length Z-score at 2 Months	1.31	0.98, 1.75	0.072

Roy SM, Chetal A, Mendiratta P, Xaio R, Dawood R, Mitchell JA, et al. Body Mass Index (BMI) Trajectories in Infancy Differ by Population Ancestry and May Precede Disparities in Early Childhood Obesity. J Clin Endocrinol Metab. 2015:100:1591-99
Wells JC, Chomtho S, Fewtrell MS. Programming of body composition by early growth and nutrition. Proc Nutr Soc. 2007;66:423-34
Wells JC. Do we need ethnic-specific body composition reference standards in childhood? Ann Hum Biol. 2014;41:1-14. Epub 2013/08/28. doi: 10.3109/03014460.2013.807877. PubMed PMID: 23980740.
Wells JC, Chomtho S, Fewtrell MS. Programming of body composition by early growth and nutrition. Proc Nutr Soc. 2007;66:423-34. Epub 2007/08/31. doi: S0029665107005691 [pii]. PubMed PMID: 17764592.

***Appendix P**:*

Hawkes CP, Zemel BS, Kenny LC, Kiely M, Sikora K, Caulfield MP, Argon Y, Reitz RE, McPhaul MJ, Grimberg A, Murray DM. The relationship between IGF-I and –II and body composition at birth and over the first 2 months of life.

Pediatric Endocrine Society, September 2017 (Poster Presentation)



Appendix Q

Junior Investigator Pilot Grant Application

UNIVERSITY OF PENNSYLVANIA

CHILDREN'S HOSPITAL OF PHILADELPHIA
CLINICAL & TRANSLATIONAL SCIENCE AWARD
CLINICAL TRANSLATIONAL RESEARCH CENTER (CTRC)

Junior Investigator Pilot Grant Program (JIPGP) – FY 2015

APPLICATION COVER PAGE

Project Title: Growth hormone sensitive short stature in childhood; A novel multisystem approach to diagnosis and characterizing the effects of treatment

Principal Investigator: Colin Hawkes	Dept./Div. Pediatrics / Endocrinology
Co-Investigator: Adda Grimberg	Dept./Div. Pediatrics / Endocrinology
Co-Investigator: Babette Zemel	Dept./Div. Pediatrics / Gastroenterology, Hepatology and Nutrition

Amount Requested: $19,998

Please check application type:

☐ Project Grant
☒ New Investigator Grant
☐ Innovation Grant

APPROVAL

	Michael Levine	02/26/15
Department Chair (or designee*/ title)	(Print name)	Date

* Usually division or section chief or department head

PROJECT DESCRIPTION

ABSTRACT:

Current guidelines for evaluating children with possible growth hormone deficiency (GHD) include monitoring growth, measuring growth factors and performing growth hormone (GH) stimulation testing. This approach has poor specificity for disease and only half of children subsequently treated with GH respond to treatment. GH treatment costs approximately $30,000 to 50,000 per patient per year. GH affects many physiological parameters other than growth, and these effects are the indications for treatment of adult GHD. Muscle mass, strength, body fat, echocardiographic features, vascular endothelial function and lipid profiles are affected by GHD and improved with GH treatment. The aim of this pilot study is to generate preliminary data for a larger study to determine if children who will respond to GH treatment have 1) baseline characteristics that predict response, 2) changes in these characteristics within 3 months of treatment that also predict response, and 3) characteristics after one year of treatment that more closely resemble untreated non-GHD controls. This pilot study will include 30 patients followed prospectively for one year.

Background

Most patients referred for evaluation of short stature have either constitutional delay of growth and puberty (CD) or idiopathic short stature (ISS).[1] Distinguishing these two diagnoses from isolated growth hormone deficiency (GHD) is difficult, as tests have poor sensitivity and specificity. Current recommendations include consideration of growth, bone age, and levels of insulin-like growth factor I (IGF-I), and IGF protein-3 (IGFBP-3) to screen for GHD, followed by a confirmatory growth hormone stimulation test (GHST).[2, 3]

Growth response to growth hormone (GH) treatment is extremely variable,[4] and 30-50% of currently treated patients do not respond.[4] GH generates almost $2 billion in annual sales revenue,[5] and costs up to $50,000 per patient per year.[6] The GHST has a central role in the decision to prescribe GH. However, up to 50% of normally growing children will be misclassified by these tests as having GHD, depending on the threshold and stimulus used.[7] Furthermore, peak stimulated GH concentration on these tests does not correlate with growth response to GH treatment,[8-10] and most children will pass a GHST if repeated when they reach adult height.[11]

GH mediates many non-growth related effects that guide treatment in adult GHD, but are largely ignored in the evaluation of pediatric GHD or monitoring of treatment.[12] The multisystem phenotype of adult GHD is well described. This includes reductions in lean body mass, exercise tolerance, fractional shortening of cardiac myocytes and high-density lipoprotein (HDL) as well as increases in fat mass, central adiposity, intima media wall thickness, triglycerides and low-density lipoprotein (LDL).[13] The effects of GH treatment on each of these systems may be dose-dependent,[14] but whether or not adverse effects of partial deficiency on each of these systems correlates with severity of deficiency in children is not known.

The interaction between GH and body composition, cardiac function and bone mineral density in pediatrics warrants further investigation. These may have significant implications for children with undiagnosed GHD, both for diagnosis and for monitoring response to therapy. Fluid retention, a known complication of GH treatment even in normal children,[15] complicates measurement of muscle mass using indirect measures such as dual-energy x-ray absorptiometry (DXA), or air displacement plethysmograpy. Pairing these methods with a reliable measure of total body water, such as deuterium oxide, would allow more detailed characterization of changes in muscle mass with GH therapy.

Specific Aims

This pilot study will generate the preliminary data necessary to conduct a more definitive study aimed at improving the identification of children who will have a positive growth response to GH treatment, and will provide information on the detailed physiological responses to GH treatment both in children who have a growth response to treatment, and those who do not. We will conduct a 1-year prospective, longitudinal study of children with short stature treated with GH, and untreated controls with similar stature. Four groups will be identified based on treatment and growth in the first year: Responders, Non-responders, Constitutional Delay (CD) and Idiopathic Short Stature (ISS) (Figure 1).

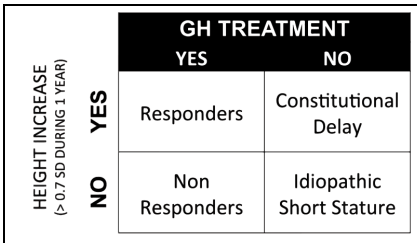


Figure 1: Diagram showing the categorization of patients based on GH treatment status and growth during one year of observation. After 1 year of treatment, responders grow >0.7 SD, non-responders do not. After 1 year of observation, children with constitutional delay (CD) grow >0.7 SD, but idiopathic short stature (ISS) do not.

Physiological characteristics measured in this study are listed in Table 1, and these will be measured at baseline, 3 months and 1 year.

Hypothesis 1: Responders, compared with non-responders, ISS and CD, will have physiological characteristics (e.g. increased body fat, altered lipid profile, reduced brachial artery reactivity) at baseline that are predictive of a significant response to GH therapy.

Hypothesis 2: Responders, compared with non-responders, ISS and CD, have changes in physiological characteristics within 3 months of treatment that predict their response to therapy.

Hypothesis 3: Following one year of GH treatment, responders, compared to non-responders, ISS and CD, have physiological characteristics that resemble untreated GH sufficient controls.

Preliminary Data

Over the past six years, there have been 1301 GHSTs performed at CHOP, and 600 (46%) of these patients were subsequently treated with GH. Many physiological parameters have been shown to respond to GH treatment, but most studies do not include a positive growth response to treatment and utilize poorly specific stimulation testing to categorize patients as having GHD.

- *Body composition:* Children with GHD have reduced lean body mass and increased fat mass at diagnosis, with a change in body water within 6 weeks of treatment possibly correlating with growth response to treatment.[16-21] No study has used multiple modalities of assessing body composition[22] to differentiate changes in body water from muscle mass or concurrently evaluated muscle function (i.e. strength). Adults with untreated childhood-onset GHD have reduced knee extensor muscle strength, and this improves with treatment.[23]
- *Bone Mineral Density:* Height Z-adjusted areal bone mineral density and volumetric lumbar spine bone density is lower in children with ISS and GHD than age and sex-matched reference ranges,[18] but the response of these parameters within one year of treatment is variable.[18, 24]
- *Cardiac and Vascular Endothelial Function:* Children with GHD have lower left ventricular (LV) mass, and functional changes in myocardial contractility.[25] LV mass changes are seen within 3 months of GH treatment.[25-27] Carotid intima media thickness may also be increased in children with GHD.[28] Endothelial function is impaired in adult GHD and responds to treatment.[29]
- *Other Markers:* Lipid profile,[30, 31] leptin,[32] fasting insulin,[16, 33] IGF-I,[34, 35] IGFBP-3,[36] IGFBP-2,[37] Homocysteine, Tumor Necrosis Factor Alpha,[38] and fibrinogen[38] may be altered in children with GHD.
- *Genetic Markers:* Next generation sequencing has been used to identify variants associated with short stature. The correlation of these loci with GH responsiveness is not known.[39-41]
- *Dermal Thickness:* Skin thickness and elasticity are reduced in children with GHD, and these may improve with treatment.[42] This effect is also seen in adults.[43]

Subjects and Recruitment

Eligibility criteria: Height-for-age <2 SDS, IGF-I <-1SD, Tanner stage 1 or 2, GHST performed. *Exclusion criteria*: Comorbidities or medications that may affect growth. Families will be invited to participate in the study on the day of GHST. Children subsequently not treated with GH will also be eligible as controls.

Methods

Children meeting inclusion criteria will be followed for one year. They will be evaluated at the CTRC Nutrition Core Lab at baseline, three months and at one year. The components of each visit are listed on Table 1. Additional samples will be collected at each visit for additional analysis supported by future grant applications.

Analysis

Patients will be divided into four groups, based on their GH treatment status and their growth response to the first year of GH treatment (Figure 1). The goal of this

Table 1: Study Protocol. All measured at baseline, 3 months and 1 year (except *baseline only or **baseline and 1 year only)

Laboratory Studies
Lipid profile, Bone markers (calcium, phosphorus, parathyroid hormone, urinary calcium:creatinine), Growth Factors (IGF-I and IGF-II).
*DNA Banking (Center for Applied Genomics), Zinc
**Ultrasensitive LH
Detailed Anthropometry
Muscle Strength: Hand grip dynamometry, knee kick, jump (force plate).
Body Composition
Dual-energy x-ray absorptiometry
Air displacement plethysmography
Bioimpedence absorptiometry
Peripheral quantitative CT
Deuterium dilution (GH untreated at baseline only, GH treated at baseline, 3 months and 1 year)
Cardiac Echo
Endothelial Function: Carotid intima media thickness, Brachial Artery Reactivity Testing, Pulse Wave Velocity.

pilot study is to estimate effect sizes that will be used to power a larger prospective study. This will involve estimating group means and standard deviations, mean analyses to assess group differences and identify potential interactions that might be assessed in our future prospective study.

For the analyses listed below, each characteristic will be included as the dependent variable in a logistic regression model that includes an indicator for variable of treatment and response. We do not anticipate achieving statistical significance in this pilot study, so our primary assessment will be of the fitted group means and estimated standard deviation.

Comparisons performed in multisystem parameters will be as follows:

H1: Baseline parameters between responders and a) non-responders, b) CD and c) ISS.

H2: Change from baseline to 3 months in multisystem parameters between responders and a) non-responders, b) CD and c) ISS.

H3: Multisystem parameters of responders at 1 year will be compared with a) non-responders, b) CD and c) ISS at one year.

Study Feasibility and CTRC Resources Requested

Approximately 18 children undergo GH stimulation testing each month, over half of whom are expected to meet eligibility criteria. The evaluations included in the study protocol are estimated to require 2.5 hours in total, and the majority of patients will be aged 7-12 years. CTRC resources requested are: **Nursing** (phlebotomy), **Nutrition** (anthropometry, body composition), **Cardiology** (echocardiography, brachial artery reactivity testing), **Biostatistics**, **Laboratory, Informatics** (database and CRF development).

Significance and the need for Junior Investigator Pilot Grant Program funds

This project will be the most comprehensive longitudinal evaluation of children with presumed GHD performed, and has potential to improve clinical care of children with short stature. The integration of multiple methods of body composition analysis, bone health, echocardiography, and metabolic outcomes will generate pilot data for accurate calculation of sample sizes and power analyses in support of a larger study to test the hypotheses listed above. Improving our understanding of the non-growth related effects of GH in pediatric patients could have significant implications for patients, society and future research. We anticipate that this work will improve identification of GH responders, and reduce exposure of non-responders to unnecessary risk, and society to the cost.[44-48]

Additional avenues of future study have been planned. DNA will be bio banked for future analysis in collaboration with the CHOP Center for Applied Genomics, using genomic data collected by this center on >45,000 children as controls. We anticipate that this collaboration will potentially identify genetic markers of GH responsiveness, either in this pilot cohort or in the larger subsequent study. Serum samples will be stored on each patient for future analysis of markers of GH responsiveness and the effect of treatment. These are exciting and novel future areas of research that will be supported by data and specimens collected, as well as experience gained, in completing this pilot study.

Timetable

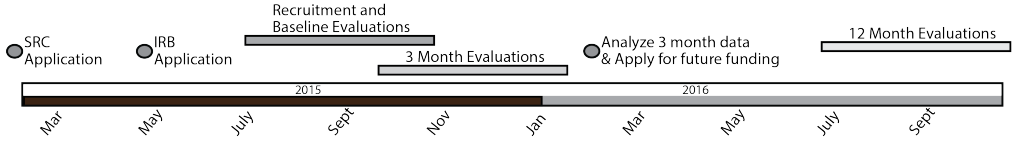


Figure 2: Proposed study timetable.

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